

# **Insights into Neural Crest Evolution**

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## ABSTRACT

Neural crest cells are unique to vertebrates and essential to the development and evolution of the craniofacial skeleton. Using a combination of DiI cell lineage tracing, transcriptomics, and analysis of key transcription factors of the Sox Family, I examined neural crest development in the sea lamprey, *Petromyzon marinus*, as the most basal extant vertebrate from which it is possible to get embryos. The results have uncovered distinct cranial and trunk neural crest subpopulations along the anterior-posterior axis of the lamprey embryo, with a clear separation between the two. However, no evidence of the presence of an intermediate vagal neural crest population was uncovered. Comparing cranial neural crest genes between lamprey and chick, either by examining individual candidate genes or whole genome transcriptome analysis, reveals significant changes in the cranial neural crest gene regulatory network of lamprey compared with chick. In particular, the lamprey cranial neural crest is “missing” several gnathostome cranial crest genes. We speculate that these may underlie the evolutionary divergence of craniofacial development between jawed and jawless vertebrates. Despite the absence of vagal neural crest, DiI-labeling shows that trunk neural crest-derived cells, likely homologous to mammalian Schwann cell precursors, contribute to the lamprey enteric nervous system, potentially representing the most primitive form of neural crest cells contribution to the ENS. Finally, I characterized key members of the Sox Family (Sox B-F) due to their importance in neural crest specification in other species. In comparative studies of the SoxC genes (Sox4, Sox11, and Sox12) in both lamprey and *Xenopus*, I found similar expression patterns and a novel key role in early neural crest specification, suggesting a conserved role of the SoxC genes amongst vertebrates. Taken together, this work represents important progress in characterizing the early evolution of the neural crest in vertebrates and its role in the transition from jawless to jawed vertebrates.

## PUBLISHED CONTENT

- 1) \*Haming, D., \*Simoës-Costa, M., **Uy, B.**, Valencia, J., Sauka-Spengler, T., Bronner, M.E., (2011) Expression of Sympathetic Nervous System Genes in Lamprey Suggests Their Recruitment for Specification of a New Vertebrate Feature. PLoS ONE 6(10): e26543. doi:10.1371/journal.pone.0026543
- 2) **Uy, B.**, Simoes-Costa, M., Sauka-Spengler, T., and Bronner-ME. (2012) Expression of Sox genes in lamprey development. Int. J. of Dev. Biol. 56: 377 – 383. doi: 10.1387/ijdb.113416bu
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- 4) **B.R. Uy**, M. Simoes-Costa, D.E. Koo, T. Sauka-Spengler, M.E. Bronner, (2015) Evolutionarily conserved role for SoxC genes in neural crest specification and neuronal differentiation. Dev. Biol., 397 (2015), pp. 282–292 doi: 10.1016/j.ydbio.2014.09.022

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	iii
ABSTRACT.....	iv
PUBLISHED CONTENT .....	v
TABLE OF CONTENTS .....	vi
LIST OF FIGURES .....	vii
CHAPTER I: <b>INTRODUCTION</b> .....	1
CHAPTER II: <b>FATE MAPPING AND TRANSCRIPTOMICS OF NEURAL CREST SUBPOPULATIONS</b> .....	13
I.    EXPRESSION OF SYMPATHETIC NERVOUS SYSTEM GENES IN LAMPREY SUGGESTS THEIR RECRUITMENT FOR SPECIFICATION OF A NEW VERTEBRATE FEATURE.....	14
II.   PROGRESSIVE REGIONALIZATION OF THE NEURAL CREST DURING VERTEBRATE EVOLUTION VIA ELABORATION OF GENE REGULATORY CIRCUITRY.....	38
CHAPTER III: <b>ANCIENT EVOLUTIONARY ORIGIN OF THE VERTEBRATE ENTERIC NERVOUS SYSTEM FROM NEURAL CREST-DERIVED SCHWANN CELL PRECURSORS</b> .....	63
CHAPTER IV: <b>NOVEL ROLE OF SOX FACTORS IN NEURAL CREST FORMATION</b> .....	81
I.    EXPRESSION OF SOX GENES IN LAMPREY DEVLOPMENT.....	82
II.   EVOLUTIONARILY CONSERVED ROLE FOR SOXC GENES IN NEURAL CREST SPECIFICATION AND NEURONAL DIFFERENTIATION.....	105
CHAPTER V: <b>CONCLUSION</b> .....	141
APPENDIX A: A FATE-MAP FOR CRANIAL SENSORY GANGLIA IN THE SEA LAMPREY.....	155
BIBLIOGRAPHY .....	196

## LIST OF FIGURES

<i>Number</i>	<i>Page</i>
1. Lamprey as a model organism .....	12
2. DiI labeling of lamprey neural crest cells reveals absence of sympathetic ganglia during embryonic development.....	30
3. Expression of the transcription factor Phox2 in embryos of <i>P. marinus</i> .....	31
4. Expression of the helix-loop-helix transcription factor Hand in lamprey embryos .....	32
5. Expression of Ascl1 during embryonic development of the lamprey .....	33
6. DiI-labeling along the anterior-posterior body axis reveals distinct populations of cranial and trunk neural crest cells .....	56
7. Comparison of chick cranial-specific gene regulatory circuit with that of lamprey .....	57
8. Transcriptome analysis comparing lamprey cranial and trunk neural crest .....	58
9. Proposed model for evolution of neural crest subpopulations .....	59
10. Cranial Neural Crest contribute to the trigeminal ganglia .....	60
11. Trunk neural crest give rise to dorsal root ganglia and fin mesenchyme .....	60
12. Early formation of enteric neurons in the lamprey <i>P. marinus</i> .....	71
13. DiI Labeled cells in the neural tube contribute to enteric ganglia .....	72
14. Schwann cell precursors contribute to the ENS .....	73
15. Number of DiI-labeled neurons (Actub+) in 25 representative E30-35 embryos in which DiI had been injected into the neural tube at T22.5.....	74
16. 5-HT immunoreactive cells in the gut, and DiI labelling.....	75

17. DiI labeling of the caudal hindbrain population shows contributions to the branchial arches but not outflow tract or gut.....	76
18. Examining schwann cell markers in lamprey.....	77
19. Phylogenetic Analysis of lamprey Sox genes .....	96
20. SoxB Expression .....	97
21. SoxD Expression .....	98
22. SoxE Expression .....	99
23. SoxF Expression.....	100
24. Maximum Likelihood phylogenetic tree of lamprey Sox genes.....	101
25. SoxC1–C4 are expressed in the neural plate border, neural tube, and neural crest derivatives in lamprey .....	127
26. <i>Xenopus laevis</i> SoxCs exhibit overlapping expression in the neural plate, neural crest, and facial region .....	128
27. Morpholino antisense oligonucleotides efficiently disrupt target protein translation.....	129
28. Loss of SoxC1, C3, and C4 in lamprey results in loss of neural crest specifier genes Sox10 and FoxD3.....	130
29. <i>Xenopus laevis</i> Sox4 and Sox11 regulate neural crest specifier genes FoxD3, Sox10, and Twist. ....	131
30. Loss of SoxC genes in lamprey causes defects in the cranial ganglia and branchial arches .....	132
31. <i>Xenopus laevis</i> Sox4 and Sox11 knock-downs affect Neurogenin expression later in development .....	134
32. Phylogenetic analysis of the SoxC Family .....	135
33. SoxC1, C3, and C4 are expressed in premigratory neural crest and crest derivatives .....	136
34. Full Length SoxC proteins .....	137
35. Spatiotemporal development of lamprey cranial sensory ganglia .....	178
36. Cyclostome Pax3/7 subfamily proteins form a well-supported clade, separate from gnathostome Pax3 and Pax7 clades .....	180



37. Pax3/7 is a specific marker for opV placode-derived neurons .....	181
38. Fate-mapping of opV and mmV placode-derived neurons reveals two separate domains of mmV neuron precursors at late neurula stages.....	182
39. Fate-maps at E6-7 for lamprey epibranchial and lateral line placode-derived ganglia .....	184
40. Neural crest-derived cells are found in cranial sensory ganglia and along cranial nerves (presumptive Schwann cells) .....	186

*Chapter 1***INTRODUCTION**

*Neural crest cells are responsible for essential derivatives in vertebrate development*

Neural crest cells are a multipotent stem cell population that is unique to vertebrates and appears to have emerged at the base of the lineage. These cells give rise to a wide variety of derivatives, such as melanocytes, sensory ganglia, Schwann cells, craniofacial skeleton, connective tissue, chromaffin cells of the adrenal medulla, parafollicular cells of the thyroid, cells of the ventricular septum, and the aorticpulmonary septum. Because of their essential role in embryogenesis, defects in neural crest development are responsible for a variety of birth defects, diseases, and syndromes. Previous studies have employed fate mapping experiments and quail-chick heterospecific grafts to demonstrate neural crest cell fate and developmental potential along the anterior to posterior body axis (Le Douarin and Kalcheim 1999). The most anterior population is the cranial neural crest, emerging from dorsal neural tube of the posterior portion of the forebrain to the hindbrain. These cells give rise to craniofacial cartilage, bone, connective tissue, smooth muscle, neurons of the trigeminal ganglion, and glia. The next population is the vagal neural crest, which emerges adjacent to the 1<sup>st</sup> to 7<sup>th</sup> somite region. Vagal neural crest cells are essential for the formation of the enteric nervous system (Le Douarin and Teillet 1973). At the level of somites 1 through 3 are the cardiac neural crest cells, which play key roles in ventricular septum formation and aorticpulmonary septation. Beyond somite 7 is the trunk neural crest, which contributes to the formation of melanocytes, dorsal root, and sympathetic ganglia.

### *Studying Neural Crest in Lamprey*

The chordate phylum can be separated into 3 subphyla: Urochordates (which includes tunicates, like *Ciona intestinalis*), Cephalochordates (e.g. amphioxus), and the vertebrates (Figure 1A). These subphyla all share common traits such as a notochord, hollow dorsal nerve cord, pharyngeal openings, endostyle, and a post-anal tail. Vertebrates can be further separated into jawed (agnathostomes) and jawless vertebrates (gnathostomes). The neural crest emerged from a vertebrate ancestor prior to this key separation. The sea lamprey (*Petromyzon marinus*), an extant jawless vertebrate, provides a unique opportunity for studying the transition from jawless to jawed vertebrates during early vertebrate evolution. Though lamprey possess many neural crest derivatives, they lack some key characteristics that are seen in jawed vertebrates such as an opposable jaw, a sympathetic nervous system, and a ventricular septum (Figure 1B). It has been postulated by “the new head hypothesis” that the invention of neural crest and placodes allowed early vertebrates to transition from being filter feeders to a larger predatory role by providing a complex craniofacial skeleton, muscularization of the gill apparatus for larger oxygen consumption, and a more complex peripheral sensory system (Glenn Northcutt 2005) (Figure 1C).

Other than the evolutionary placement as the most basal extant jawless vertebrate that we can obtain embryos from, lamprey are also valuable due to their slow development, large number of embryos that can be extracted, and relative size. Approximately 5 hours after fertilization, lamprey embryos begin their first division, which makes them amenable to single blastomere injections which ultimately result in a lateral distribution with one side of the embryo containing the construct and the other as an internal control. Due to the slow

development, the two-cell period lasts for approximately an hour and a half, providing sufficient time to quickly introduce the desired construct or treatment. Despite these advantages, some tradeoffs are made in lamprey. They only breed during the summer, have few identified tissue specific promoters, high levels of auto-florescence of the embryo due to high yolk content, and have few functioning antibodies due to lack cross reactivity. Nevertheless, the benefits of the knowledge that this ancient organism can shed on neural crest evolution are priceless and essential to understanding the role of neural crest.

### ***Conservation of the Neural Crest Gene Regulatory Network in Vertebrates***

In all vertebrates, neural crest cells arise at the borders between neural and non-neural ectoderm. During neurulation, the neural folds elevate and form the neural tube, with a lumen that forms either during tube closure or cavitation. Neural crest cells delaminate from the most dorsal aspect of the neural tube by undergoing an epithelial to mesenchymal transition (EMT) and subsequently migrate along well established pathways to diverse sites. The gene regulatory network (GRN) underlying neural crest (NC) formation has been studied in a number of vertebrate models including chick, mouse, and zebrafish. Neural crest formation begins with inductive signals (Wnts, Bmps, Fgfs, and Delta/Notch) which establish the border between neural and non-neural ectoderm that expresses neural plate specifier genes (e.g. *Zic*, *Pax3/7*, *Msx1/2*, *Dlx3/5*) which in turn activate neural crest specifiers (*SoxE*, *FoxD3*, *Tfap2*, *Snail*, *Id*, *Twist*, and *Ets1*) which drive EMT and migration (Meulemans and Bronner-Fraser 2004, Sauka-Spengler, Meulemans et al. 2007, Betancur, Bronner-Fraser et

al. 2010). Non-vertebrate chordates do not have *bona fide* neural crest but do have many of the transcription factors within their genomes, although these often differ in their spatial and temporal expression. In the *Amphioxus*, only the neural crest specifier gene *Snail*, but not other neural crest specifiers, is expressed in the dorsal neural tube in contrast to the other neural crest specifier genes (Meulemans and Bronner-Fraser 2004). Ascidians have some population of cells that express some neural plate and neural crest specifiers genes (Jeffery, Strickler et al. 2004, Abitua, Wagner et al. 2012) and migrate from the central nervous system (Stockli et al., 2015), perhaps representing a cell population that is transitional. In lamprey, many of these genes of the NC GRN have conserved function with only small differences in temporal expression. The exception is the late neural crest specifier genes *Twist* and *Ets1*, which are not expressed in lamprey premigratory neural crest, but only at later stages. Thus, it appears as though the proximal GRN elements were in place in the vertebrate common ancestor, and have been highly conserved across all vertebrates (Sauka-Spengler, Meulemans et al. 2007), with some small regulatory changes that may account for some differences in select derivatives. However, no invertebrate chordate has multipotent neural crest cells and they lack a regulatory cascade that includes Sox family members.

### ***Essential role of Sox genes in Growth and Development***

Although the Sox genes exist in many invertebrates and vertebrates, many of the Sox genes, in particular the SoxE (Sox8,9,10) and SoxD (Sox5 and 6) members, appear to be essential in the neural crest GRN and emergence of neural crest in vertebrates, but the

Sox genes are not restricted to this role. These transcription factors possess a conserved high mobility group (HMG) that binds to WWCAAW in the minor groove, causing a conformational change in the DNA for other factors. They must complex with partner genes to regulate gene expression (Kamachi and Kondoh 2013). Sox genes were first identified for their role in sex determination and later in various other processes including neural crest specification, chondrocyte differentiation, and neurogenesis. The SoxB1s (Sox1-3) are essential in early neural plate formation and maintaining undifferentiated state of neural precursors. In contrast, the SoxCs (Sox4, 11, and 12), single exon genes that are present in all metazoans contain a C-terminal transactivation domain, are expressed in a complimentary fashion in post mitotic and differentiated neurons. They are associated with proneural basic helix loop helix genes such as Neurogenin1, Neurogenin2, and Mash that initiate neurogenesis by inducing stem cells to exit the cell cycle (Bergsland, Werme et al. 2006). Recently, the SoxCs have emerged as an important group due to their activity in various forms of cancer due to the Sox family genes' ability to modulate Wnt/ $\beta$ -catenin and TGF- $\beta$  signaling cascades. Sox4 has been shown to induce EMT in cell culture and in growth of tumor such as nasopharyngeal carcinoma. Interestingly, Sox4 can operate as both an oncogene and tumor suppressor. In cases of glioblastoma multiforme (GBM), high Sox4 levels are a positive prognostic factor for survival by inhibiting growth through G0/G1 cell cycle arrest via activation of p53 and down regulation of AKT1 (Zhang, Jiang et al. 2014). Sox11 also shows positive prognostic factors for GBM patients. Sox11 is seen in many B-cell lymphoma (mantle cell lymphoma) due to Sox11's ability to activate Pax5, which regulates Blimp1 (Vegliante, Palomero et al. 2013). Although the function of SoxC genes has been explored during neurogenesis, cancer, and eye development, little

attention has been paid to their role in early embryonic development in any species, likely due to the fatal nature of early knockout studies. If examined earlier in both jawless and jawed vertebrates, these genes may play a role in regulation of neural crest specification.

By comparing gene function between jawed and jawless vertebrates, we can gain insight into their evolutionary history and origin via duplication events. Through Ohno's 2R hypothesis, gnathostomes are thought to have undergone two rounds of genome wide duplication, at least one and possibly both of which are thought to have occurred prior to the divergence of jawed and jawless vertebrates (Dehal and Boore 2005). With gene duplications, a redundant copy of the gene is generated, allowing for modification and a potential for a greater genetic diversity. From these duplicated genes, selective pressure results in possible emergence of new functions (neofunctionalization), redistribution of functionality (subfunctionalization), or loss of the gene from the genome. It is thought that many paralog genes that were duplicated either through genome wide duplication or gene specific duplications have been lost, with only a subset retained. Therefore, not all genes in mammals will contain four paralogs. Given their importance, the Sox Family represents a prime candidate family to study duplication events prior to the separation of jawed and jawless vertebrates.

### ***Neural Crest Regionalization in Vertebrates***

In addition to examining gene duplications, comparison between jawed and jawless vertebrates may reveal key differences in regionalization of neural crest along the body axis. Cranial neural crest cells are essential for craniofacial development. In humans, branchial



arches 1 through 6 are derived from neural crest and mesoderm. Neural crest cells from rhombomeres 1 and 2 contribute to the cartilage, bone, and sensory elements of cranial nerves V, VII, IX, and X. The neural crest of the first branchial arch contributes to Meckel's cartilage, the mandible, malleus, incus, and the sphenomandibular ligament, and cranial nerves V<sub>2</sub>/V<sub>3</sub>. In Treacher Collins Syndrome, the neural crest of the first branchial arch fails to migrate, leading to mandibular hypoplasia and facial abnormalities, such as cleft palate and cleft lip. Trunk neural crest, which migrate along a dorsolateral route to form melanocytes and ventral pathways to contribute to neuronal and glial lineages, share some common neural crest derivatives with other axial levels. However, *in vivo*, grafts from the trunk level to cranial regions have demonstrated that trunk neural crest lack the ability to produce skeletal elements except under extremely long culture conditions (Le Douarin and Teillet 1973, McGonnell and Graham 2002). Thus, understanding the differences in regulatory elements of the cranial regions against the trunk may be essential in understanding skeletal and craniofacial development.

### ***Neural crest contributions to the Enteric Nervous System in Vertebrates***

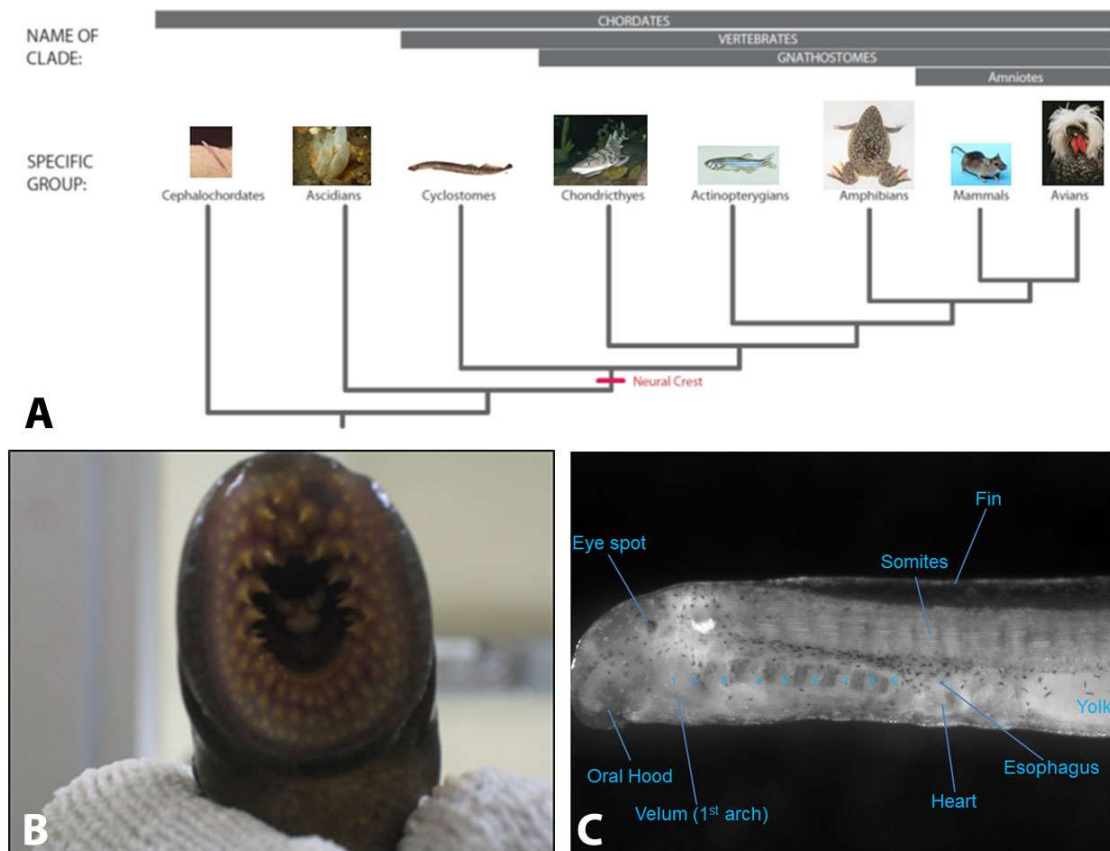
Other than cranial and trunk neural crest, the vagal neural crest produces a key derivative in jawed vertebrates: the enteric nervous system (ENS). Vagal neural crest cells first emigrate from the dorsal neural tube and migrate ventrally toward the foregut. After entering the foregut, they move caudally along the gut, forming the ENS and giving rise to two main enteric ganglia: the myenteric and submucosal plexuses. The myenteric (Auerbach's) plexus, which are located between the inner circular and outer longitudinal

smooth muscle of the muscularis propria, are important for gastrointestinal motility and sensing distention. Some ENS neurons have excitatory neurotransmitters including acetylcholine (Muscarinic type 1 receptors on smooth muscle) and substance P, while others have inhibitory neurotransmitters such as nitric oxide (NO) and vasoactive intestinal peptide (VIP). The submucosal (Meissner's) plexus, located in the submucosa directly below the muscularis mucosa, is important in blood flow regulation, secretion, absorption, and chemosensation. 5-hydroxytryptamine (serotonin; 5-HT) is essential in the activation of the peristaltic and secretory reflex. Enterochromaffin cells, thought to be endoderm derived, also have serotonin and secrete it in a paracrine manner to activate submucosal intrinsic primary afferent neurons which release calcitonin gene related peptide and acetyl choline to the myenteric plexuses which in turn control peristaltic movements. Despite inhibitory innervation from noradrenergic sympathetic nervous system and excitatory parasympathetic muscarinic cholinergic inputs, the ENS is known as the "little brain of the gut" since it is semi-autonomous in its ability to regulate the gastrointestinal tract independent of the CNS. It even contains its own rhythmic pacemaker activity generated from the intestinal cells of Cajal. In jawed vertebrates, the enteric nervous system is mostly derived from neural crest cells. Mutations in Ret result in the inability of neural crest cells to migrate along the entire extent of the gut, leading to an absence of ganglion cells known as Hirschsprung's disease (colonic aganglionosis). Hirschsprung's Disease typically affects the most distal portions of the distal sigmoid colon (recto-sigmoid) due to the inability of vagal neural crest to fully migrate and populate the colon. The defects in relaxation and peristalsis in a distal region causes a massive distention and expansion of the regions proximal to the defect (megacolon). Proximal portions of the intestines contain ganglia and are fully functional.

Recently, in addition to the vagal neural crest, a population of neural crest cells from the trunk referred to as “Schwann cell precursors” (SCP) has been shown to contribute to a subpopulation of neurons in the gut (Uesaka et al., 2015). SCPs were first identified associated with peripheral nerves and contributing post-embryonically to melanocytes and parasympathetic ganglia (Espinosa-Medina, Outin et al. 2014). During ENS development, trunk neural crest-derived SCP migrate along spinal nerves and contribute to a minor portion (~20%) of enteric neurons (Uesaka, Nagashimada et al. 2015). These cells are *Ret* negative and contribute to submucosal plexuses in both the small intestine and large intestine while only in the submucosal plexuses of the small intestine. All chordates have an enteric nervous system but lamprey are the first chordate to have neural crest cells.

The goal of this thesis is to understand the evolution of neural crest by examining the neural crest in a basal extant vertebrate, the lamprey, via fate mapping, transcriptomics, and GRN comparison. This provides a unique opportunity to study how the neural crest may have acquired its ability to subsume a cell type, enteric neurons, into its repertoire, an event that appeared to occur at the transition between jawless and jawed vertebrates. Through DiI labeling, my results reveal a clear regionalization of cranial and trunk neural crest subpopulations, but were unable to find a vagal neural crest population as is present in jawed vertebrates. Through comparison of the lamprey cranial neural crest gene regulatory network and transcriptome data, my results show major differences from gnathostomes with respect to expression of lamprey orthologs which may aid in the programming and formation of the craniofacial skeleton in jawed-vertebrates. Despite the lack of vagal neural crest, my work shows a contribution of trunk neural crest-derived Schwann cell precursors to the lamprey

enteric nervous system. We speculate that these SCPs may represent an intermediate cell type in enteric nervous system formation. Finally, I explored the lamprey genome and examined the conservation of an important gene family involved in neural crest specification and migration, the Sox gene family. I found a conservation of many of these genes between lamprey and other vertebrates. In addition, the SoxC family serves a key role in early neural crest specification. Using these data, I have made inroads in understanding the evolution of neural crest as it emerges in vertebrates and transitions to jawed vertebrates.



**Figure 1: Lamprey as a model organism:**

Phylogeny of select model chordates. (B) The mouth of an adult sea lamprey (*petromyzon marinus*) from the ventral view. (C) Key structures in an E16 lamprey larva.



*Chapter II***FATE MAPPING AND TRANSCRIPTOMICS OF NEURAL CREST  
SUBPOPULATIONS**

*Chapter 2 - 1*

**EXPRESSION OF SYMPATHETIC NERVOUS SYSTEM GENES IN LAMPREY  
SUGGESTS THEIR RECRUITMENT FOR SPECIFICATION OF A NEW  
VERTEBRATE FEATURE**

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**Abstract**

The sea lamprey is a basal, jawless vertebrate that possesses many neural crest derivatives, but lacks jaws and sympathetic ganglia. This raises the possibility that the factors involved in sympathetic neuron differentiation were either a gnathostome innovation or already present in lamprey, but serving different purposes. To distinguish between these possibilities, we isolated lamprey homologues of transcription factors associated with peripheral ganglion formation and examined their deployment in lamprey embryos. We further performed DiI labeling of the neural tube combined with neuronal markers to test if neural crest-derived cells migrate to and differentiate in sites colonized by sympathetic ganglia in jawed vertebrates. Consistent with previous anatomical data in adults, our results in lamprey embryos reveal that neural crest cells fail to migrate ventrally to form sympathetic ganglia, though they do form dorsal root ganglia adjacent to the neural tube. Interestingly, however, paralogs of the battery of transcription factors that mediate sympathetic neuron differentiation (*dHand*, *Ascl1*, and *Phox2b*) are present in the lamprey genome and expressed in various sites in the embryo, but fail to overlap in any ganglionic structures. This raises the intriguing possibility that they may have been recruited during gnathostome evolution to a new function in a neural crest derivative.



## Introduction

Lampreys are agnathans (jawless vertebrates) that have many essential vertebrate characteristics but lack the sympathetic nervous system and jaws. Morphologically, they resemble Cambrian era fossils [1], [2], suggesting a resemblance to the common ancestor of jawless (Agnatha) and jawed (Gnathostomata) vertebrates. Lamprey and hagfish, the only modern agnathans, are likely to be monophyletic, though there remains controversy on this point [3], [4]. As basal vertebrates, both occupy a critical phylogenetic position for understanding emergence of vertebrate traits. However, lamprey offers a significant advantage for developmental studies due of the accessibility and ease of obtaining embryos for experimental manipulation.

Neural crest cells are one of the defining features of vertebrates. This population of multipotent cells gives rise to a variety of different tissues and cell types including cartilage and bone of the facial skeleton, pigment cells, and sensory and peripheral ganglia, among other derivatives [5], [4]. The peripheral nervous system of jawed vertebrates is composed of sensory, parasympathetic, sympathetic, and enteric ganglia that form clusters of neurons that innervate peripheral structures and relay information back to the central nervous system. All of these sensory and autonomic ganglia are derived from the neural crest, together with a contribution of cranial placodes to the sensory ganglia of the head [6].

Previous studies have shown that lampreys possess neural crest cells and many neural crest derivatives [7], [8], [9], like cartilage, pigment cells, and neurons. In fact, the

gene regulatory network underlying the formation and differentiation of neural crest cells is remarkably similar to higher vertebrates [10]–[12]. Interestingly, however, lampreys lack some key neural crest structures including dentine, bone and sympathetic neurons.

The sympathetic nervous system is a branch of the autonomic nervous system, responsible for the physiological modulation of inner organs in the absence of conscious control by the central nervous system (CNS). Lampreys and hagfishes lack the chain of sympathetic ganglia chain observed in gnathostomes [13]. Instead, their sympathetic innervation comes from preganglionic fibers that extend directly to the terminal plexus, similar to what is observed in amphioxus [14]. Nevertheless, lamprey and hagfish have been reported to have scattered chromaffin-like cells along blood vessels, the heart and cloaca [15]. Although these cells have been described as analogous to postganglionic neurons [14], it is not yet clear if they connect with the central nervous system and/or represent an evolutionary precursor to the gnathostome sympathetic nervous system [15].

There are a handful of characteristic markers for sympathetic neurons, including *Phox2b*, *Ascl1* (*Ash1*), and *dHand* (*hand2*). *Phox2b* is a homeodomain transcription factor expressed in several types of neurons in the developing nervous system [16]. The bHLH transcription factor *achaete-scute homolog 1* (*Ascl1* formerly *ash1*) is a proneural gene that influences neuronal fate. *Ascl1* is expressed in some domains of the neuroepithelium of the forebrain and in precursors of sympathetic and enteric neurons [17]. *dHand* is a basic helix-loop-helix transcription factor that is essential for proliferation and noradrenergic differentiation of sympathetic neuron precursors during

development [18]. Here, we asked whether this suite of genes exists in lamprey and if so, where they were expressed.

To address this question, we isolated lamprey homologues of these genes and examined their expression patterns in embryos by *in situ* hybridization at various stages of development. The results show that all three genes are individually found in different areas of the head with only Phox2 expressed in cells at the trunk level. DiI labeling of presumptive neural crest cells failed to show a neural crest contribution to sites where sympathetic ganglia would be expected to coalesce. In contrast, DiI labeled neural crest cells contributed to both dorsal root ganglia and enteric ganglion cells of the gut. Taken together, the results raise the intriguing possibility that the transcriptional program responsible for migration to and/or differentiation within the site of sympathetic ganglion formation in gnathostomes was assembled through the recruitment of Phox2b, dHand and Ascl1 by precursors derived from the neural crest.

## Results

While it has been established that lamprey lack an organized sympathetic nervous system [14], [19], some studies suggest that scattered sympathetic neurons and chromaffin cells might be associated with blood vessels, hindgut, cloaca, and kidneys [15]. Furthermore, it has been suggested that lamprey have sympathetic innervation to the heart [13], [20], as opposed to spinal adrenergic innervation as seen in other vertebrates. To investigate the organization of the sympathetic nervous system in lamprey embryos, we examined neural crest migration in the vagal and trunk regions, and

mapped the expression pattern of markers characteristic of differentiating sympathetic neurons.

***DiI and neurofilament labeling of trunk neural crest in lamprey***

In the trunk region of gnathostomes, neural crest precursors to sensory and sympathetic ganglia migrate from the dorsal neural tube along a ventral pathway to coalesce either next to the neural tube, to form dorsal root ganglia, or further ventrally adjacent to the dorsal aorta, to form sympathetic ganglia.

To test whether lamprey neural crest cells migrate ventrally to contribute to sensory and/or sympathetic ganglia as in gnathostomes, we first performed focal injections of the lipophilic dye, DiI, into the dorsal neural tube at trunk levels. In lamprey, the neural tube forms by secondary neurulation, where a solid rod-like structure transforms into a tube whose lumen forms by cavitation. At embryonic day 5, the neural rod elevates, gradually detaching from the dorsal epithelium. The first indication of neural crest precursors occurs at this time. The head morphologically extends and becomes visible at day 6, concomitant with cavitation. Neural crest primordia at this stage appear as bulges on the dorsal aspect of the newly formed neural tube [21]. Focal injections of DiI performed at any level of the trunk dorsal neural rod prior to cavitation (day 5.5 to day 6) (Figure 1A) failed to give rise to any migrating neural crest. In contrast, similar injections into the head neural folds at similar stages resulted in labeling of the migrating crest (Figure 1A and 1B).

Accordingly, we performed DiI-labeling at later times, after cavitation at day 6.5 to day 7, by injecting dye into the lumen of the neural tube (Figure 1D). This approach resulted in labeling of migrating neural crest cells. In embryos receiving neural tube injections at day 6.5 to 7 and examined through day 34, the labeled cells contributed to several neural crest derivatives at trunk and vagal levels (Figure 1E). These include dorsal root ganglia (DRGs) (Figure 1F and 1G) and the mesenchymal cells of the fin (Figure 1H), and enteric ganglia (Figure 1I). However, no structures resembling sympathetic ganglia were observed at any stage. These results suggest that lamprey neural crest cells contribute to dorsal root ganglia but fail to condense into sympathetic-like structures during embryonic development.

To examine neuronal differentiation, we performed immunostaining using antibodies against neuronal markers. At day 16, anti-neurofilament staining was observed in the neural tube, dorsal root ganglia (Figure 1J) and also in the ventral part of the gut, likely staining enteric ganglia. Some embryos were allowed to develop until day 31, at which time the dorsal root ganglia continued to express neurofilament protein and appeared somewhat larger in size than at earlier stages (data not shown). However, at no time point did we note neurofilament staining in the vicinity of the dorsal aorta where sympathetic ganglia coalesce in gnathostomes.

***Expression pattern of transcription factors associated with sympathetic neuron differentiation in the lamprey***

In gnathostomes, several transcription factors have been implicated in sympathetic nervous system formation. These include the basic-helix-loop helix factors, dHand and Ascl1, as well as the homeodomain transcription factor Phox2b. To examine the presence and deployment of these lamprey genes during neural crest development, we cloned fragments of lamprey Hand, Phox2 and Ascl1 orthologues using 5'RACE and determined their expression patterns by *in situ* hybridization.

Phylogenetic analysis places the putative Phox2 lamprey ortholog at the base of the branch containing gnathostome Phox2a and Phox2b genes (Figure 2A). This suggests that the duplication event that gave rise to these paralogs took place after cyclostomes diverged from the vertebrate lineage. Expression of lamprey Phox2 was first observed in hindbrain motor neurons and the ventral branchial mesenchyme at day 7 and 8 (Figure 2B and C). By day 10, there was additional expression in the epibranchial ganglia and cranial nerves (Figure 2D). Expression of Phox2 was also observed in individual cells in a medial stream reminiscent of cells migrating posteriorly (Figure 2D). On day 12, expression was maintained in the hindbrain, cranial nerves and ventral mesenchyme (Figure 2E and 2H). Moreover, the expression domain expanded to include the anterior epibranchial ganglia (arrow on Figure 2E). In the trunk, the medial expression first observed on day 10 appeared to expand posteriorly (Figure 2F and I). At later stages,

Phox2 transcripts also were detected in cells surrounding the yolk (E14, arrow on Figure 2G) and in the notochord.

The Hand ortholog isolated from lamprey clusters to the base of the branch that contains both the D-Hand and E-hand gnathostome genes in our phylogenetic analysis (Figure 3A). Lamprey Hand is first observed at day 5 in the bilateral precursors that form the cardiac field (data not shown). From day 7 to day 10, there is additional staining visible in the anterior portion of the ventral mesenchyme (Figure 3D and 3F). At day 12, in addition to the heart, the entire ventral mesenchyme that surrounds the endostyle and the notochord expresses Hand (Figure 3D and 3F). Hand transcripts were also detected at day 14 in the cardiac ganglia (Figure 3D and 3E) and in the posterior mesoderm (Figure 3G).

The lamprey Ascl1 fragment was within the branch that contains the Ascl1 gnathostome orthologues (Figure 4A). Expression of Ascl1 was first noted at day 6, with very faint expression observed in the pituitary gland (Figure 1B). This pattern was maintained at day 7, accompanied by additional expression in the lens (Figure 4C). By day 10, Ascl1 was upregulated in the anterior lip mesoderm (day 10) and by day 12, transcripts also were detected in the VI cranial ganglion (Figure 4D). From day 11 onward, Ascl1 also was expressed faintly in the notochord (Figure 4G).

In addition, analysis of the expression of these three transcription factors in lamprey juveniles through *in situ* hybridization performed in tissue slices failed to reveal any co-expression in ganglionic structures (data not shown). Thus, we were unable to identify a structure resembling sympathetic ganglia in the lamprey embryo in which

Hand, Ascl1 and Phox2 were co-expressed. Our results, in concert with the neurofilament and DiI data, demonstrate that the lamprey does not possess a sympathetic nervous system analogous to that in gnathostomes. We were also unable to identify any cells that might represent precursors to sympathetic postganglionic neurons of cyclostomes.

## **Discussion**

We analyzed the expression patterns of three known marker genes of the gnathostome sympathetic nervous system for their deployment in lamprey embryos. At 9 and 11 days after fertilization, we found expression of Hand, Ascl1 and Phox2 in the head region, with only Phox2 showing additional expression in a stream of cells that migrate toward the trunk. These cells may correspond to lateral line cells derived from ectodermal placodes. In ganglia, we see only Hand expressed in the cardiac ganglia and Phox2 in the epibranchial ganglia.

Interestingly, there was no overlapping expression of these “sympathetic” genes in any domain in the embryo. Consistent with this marker analysis, we found no contribution of DiI-labeled neural crest cells to structures adjacent to the dorsal aorta, where sympathetic ganglia arise in gnathostomes, despite finding robust labeling of other neural crest derivatives like dorsal root ganglia and enteric neurons. In addition, the absence of neuronal staining with a neurofilament antibody supports the lack of sympathetic ganglia, while revealing the presence of dorsal root and enteric ganglia. Taken together, our results suggest that the lamprey does not have a definitive sympathetic nervous system.



In gnathostomes, *Ascl1* is generally required for development of autonomic neurons, with expression initiating earlier than *Phox2b*. *Phox2b* is also required for autonomic neurogenesis and, in a feedback loop, is required for maintenance of *Ascl1* expression [22]. *Ascl1* induces expression of pan-neuronal genes in neural crest precursor cells of the peripheral nervous system, but does not specify subtype specific expression of tyrosine hydroxylase (TH) or dopamine- $\beta$ -hydroxylase (DBH), the enzymes responsible for the catalyzing synthesis of the neurotransmitter, nor-epinephrine [23]. Loss-of-function of *dHand*, another determinant of the sympathetic lineage, blocks neural crest cell differentiation into noradrenergic neurons, whereas its over-expression upregulates *Phox2b*, TH and DBH. Expression of *dHand* depends on *Phox2b*, but not *Ascl1* [22].

Classical literature regarding the lamprey sympathetic nervous system is scarce and contradictory. Lampreys possess several neurotransmitters, including acetylcholine and noradrenaline, in the central nervous system. In addition, sequenced fragments from lamprey DNA reveal the presence of two adrenergic receptors [24] that, in gnathostomes, are the most abundant in the sympathetic nervous system. While this suggests the presence of sympathetic activity in cyclostomes, ganglionic structures are absent in the trunk and tail of the lamprey. Only a sympathetic ganglion formed by small intensely fluorescent cells (SIF cells) has been described adjacent to the adult heart [25], [26]. It is possible that such a ganglion would be innervated by the vagus nerve, which is cardio-inhibitory in all vertebrates, with the exception of the cyclostomes [27]. Additionally, it has been suggested that cardiovascular function is controlled by chromaffin cells, located on the wall of blood vessels, kidney and urogenital ducts[28].

SIF cells and chromaffin cells are closely related, and SIF cells are considered to be the intermediate in morphology between chromaffin cells and sympathetic neurons [28]. One intriguing possibility is that there may have been a shift from chromaffin and SIF cells to sympathetic neurons during gnathostome evolution, such that chromaffin and SIF cells represent the evolutionary precursor to sympathetic neurons. These three cell types are closely related lineage-wise, sharing a sympathoadrenal progenitor which co-expresses markers characteristic of both chromaffin cells and sympathetic neurons [28], [29].

Our DiI labeling experiments show a discrete neural crest stream that migrates towards the heart (the cardiac neural crest; data not shown) which could be the source of the progenitors that give rise to the cardiac SIF cell aggregation [26]. Indeed, there is expression of *Hand* in ganglia adjacent to the heart (figure 3F), although *Phox2* is not present in the same structures. However, at the stages examined, we failed to observe migratory precursors that might give rise to the scattered chromaffin cells that are said to occur throughout the lamprey body.

In conclusion, our results indicate that no structures homologous to sympathetic ganglia arise during lamprey embryogenesis. While lamprey may possess a blueprint for the sympathetic nervous system composed of chromaffin and SIF cells, a more detailed analysis of the molecular identity of such cells would be necessary to establish them as true phylogenetic precursors of sympathetic neurons. Thus, our data suggests that sympathetic chain neurons are a gnathostome innovation, and that the recruitment of

Phox2, Hand and Ascl1 into a new gene battery allowed for the emergence of this new neural crest derivative.

## **Materials and Methods**

### ***DiI labeling in lamprey embryos***

5 –7 day old embryos were dechorionated in 0.1xMMR and placed into agarose-coated petridishes. DiI solution (0.5 µg/µl, prepared in 0.3M sucrose) was filled into glass needles and injected into distinct neural crest population or into the entire neural tube. Embryos were analyzed for the injection into a discrete location or into the neural tube by fluorescence microscopy. Embryos were raised in petri dishes containing 0.1xMMR at 18°C and the migration of DiI stained cells was analyzed every day. Once the embryos had reached the desired stage they were fixed using 4%Paraformaldehyde in PBS at room temperature for 1hr.

### ***Obtention of lamprey orthologs through 5' Rapid Amplification of cDNA ends (RLM-5' RACE)***

Orthologs of Phox2b, dHand and Ascl1 were identified by bioinformatic survey of the lamprey genomic sequences and cloned using RACE. Total RNA was extracted from embryos using the Ambion: RNAqueous kit. RLM-5' RACE was conducted on the total mRNA in accordance with Invitrogen: GeneRacer Kit. Total RNA was dephosphorylated through Calf Intestinal Phosphatase (CIP) treatment, decapped via Tobacco Acid Pyrophosphatase (TAP), ligated with the GeneRacer RNA oligo, and finally reverse

transcribed using random hexamer priming to form the cDNA template. The genes of interest were amplified using touch down PCR and cloned with TOPO TA Cloning Kit (Invitrogen).

### ***Immunostaining of lamprey embryos***

Immunostaining of lamprey embryos was performed as previously described [30]. Neurofilament (NF-M) antibody was used 1:200 in blocking solution. As a secondary antibody Alexa 488 anti mouse IgG2a was used 1:1000 in blocking solution. Sections were degelatinized in 42°C PBS for 10 minutes and washed with PBSTr 2 times for 5 minutes. Afterwards the sections were blocked in 10% goat serum in PBSTr at 4°C for 5 hrs. The blocked sections were incubated overnight at 4°C with the neurofilament (NF-M) antibody 1:200 in blocking solution. To remove unbound antibody the sections were washed 5 times for 10 minutes with PBSTr. As a secondary antibody Alexa 488 anti mouse IgG2a 1:1000 in blocking solution was used for 2 hrs at room temperature. Unbound secondary antibody was washed of 3 times for 10 minutes in PBSTr followed by 2 washes for 10 minutes in PBS. For mounting, sections were dipped into distilled water a few times and mounted with Permaflour.

### ***Whole-mount in situ hybridization on lamprey embryos***

Whole-mount lamprey *in-situ* was performed as previously described [21]. Plasmid DNA templates used for digoxigenin-labeled RNA probes were Hand, Ascl1 and Phox2.

Embryos fixed in MEMFA for 1 hr at room temperature. For detection of transcripts, embryos were bleached with a 10% hydrogen peroxide solution for 10 minutes, washed with PBSTw (DEPC) 3 times for 5 minutes, and were treated with 20 µg/ml proteinase K in PBSTw (DEPC) for 10 minutes. Following this incubation the proteinase K solution was replaced with 2 mg/ml glycine in PBSTw (DEPC) for 10 minutes. After this treatment the embryos were postfixed with 4% PFA for 20 minutes. Afterwards the embryos were pre-hybridized for 3 hrs in hybridization solution at 70°C, and incubated in hybridization solution containing 1–10 µg/ml labeled RNA probe for 16 hrs at 70°C. To remove unbound probe the embryos were washed twice for 15 minutes with hybridization solution at 70°C and then 4 times for 45 minutes. Afterwards the embryos were washed with hybridization solution and MABT 1:1 at 70°C for 30 minutes followed by four washes in MABT only at room temperature for 30 minutes wash. Blocking of the embryos was done for 4 hrs in blocking solution. The embryos were subsequently incubated with the Anti-DIG-AP antibody 1:2000 in blocking solution overnight at 4°C. To remove unbound antibody, embryos were washed with MABT for 2 times 5 minutes, 2 times 30 minutes, 6 times 1 hr and an overnight wash at 4°C. For the color reaction the embryos were adjusted to NTMT buffer by 4 washes for 15 minutes. For the color reaction we used BM purple. The embryos were incubated in BM purple, covered from light, until the desired staining intensity was reached. To stop the color reaction the embryos were washed in PBSTw 3 times for 5 minutes and fixed again in 4% paraformaldehyde for 2 hrs at room temperature.

### ***Embedding and sectioning of lamprey embryos***

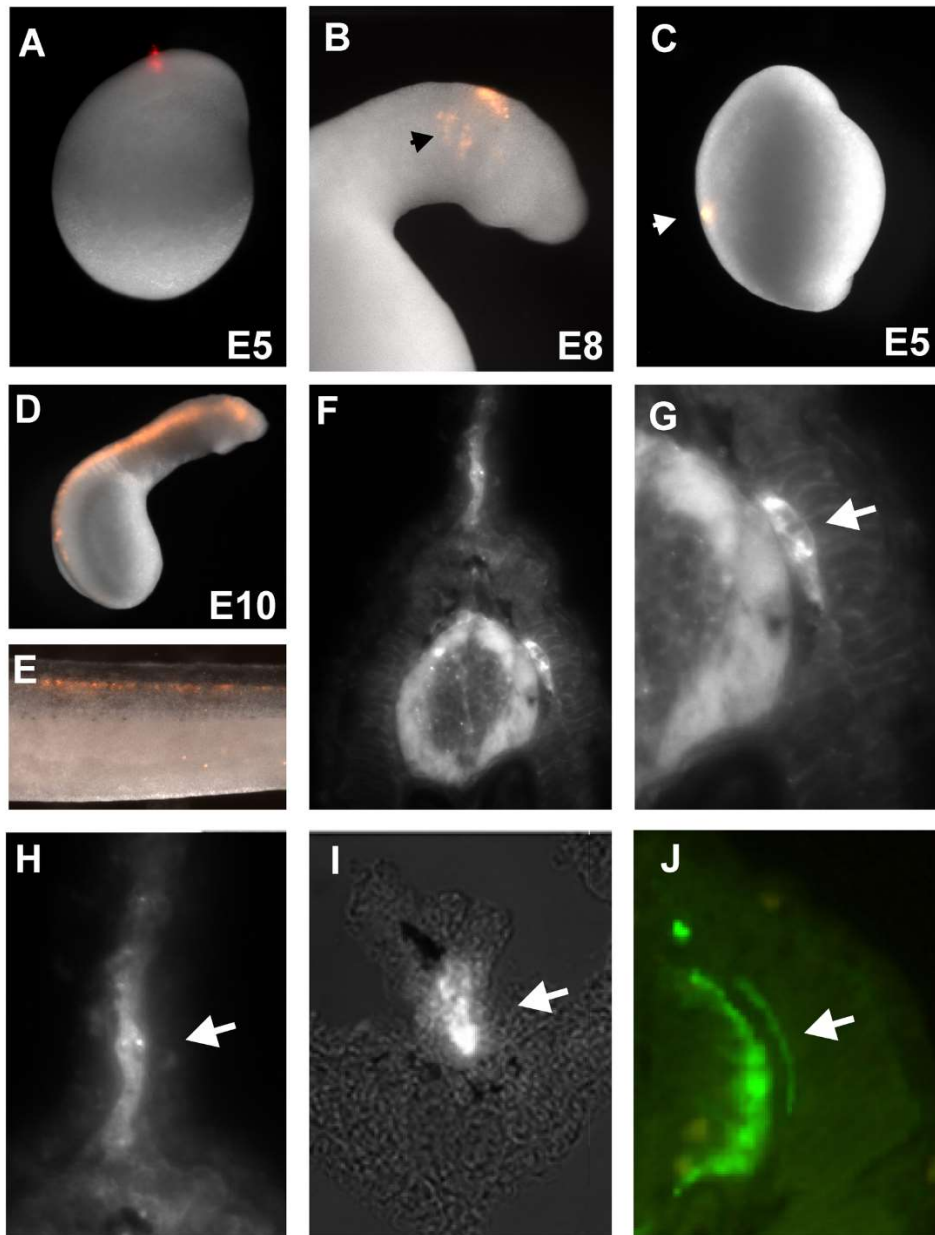
Lamprey embryos were washed with PBSTr 3 times 15 min. Subsequently they were incubated in 15% sucrose in PBS for 3 hrs at room temperature, 7.5% gelatin and 15% sucrose for 12 at 37°C, and 20% gelatin for 4 hrs at 37°C. Subsequently embryos were positioned in 20% gelatin and frozen with liquid nitrogen. Embryos were sectioned at 8 – 10 µm with a Microm HM550 cryostat.

### ***Phylogenetic Analysis***

Alignments were built with the coding sequences retrieved from GenBank. Neighbor Joining (NJ) tree were constructed using ClustalX protocol from the DNA STAR package. The trees were visualized using Tree View v. 0.5.0.

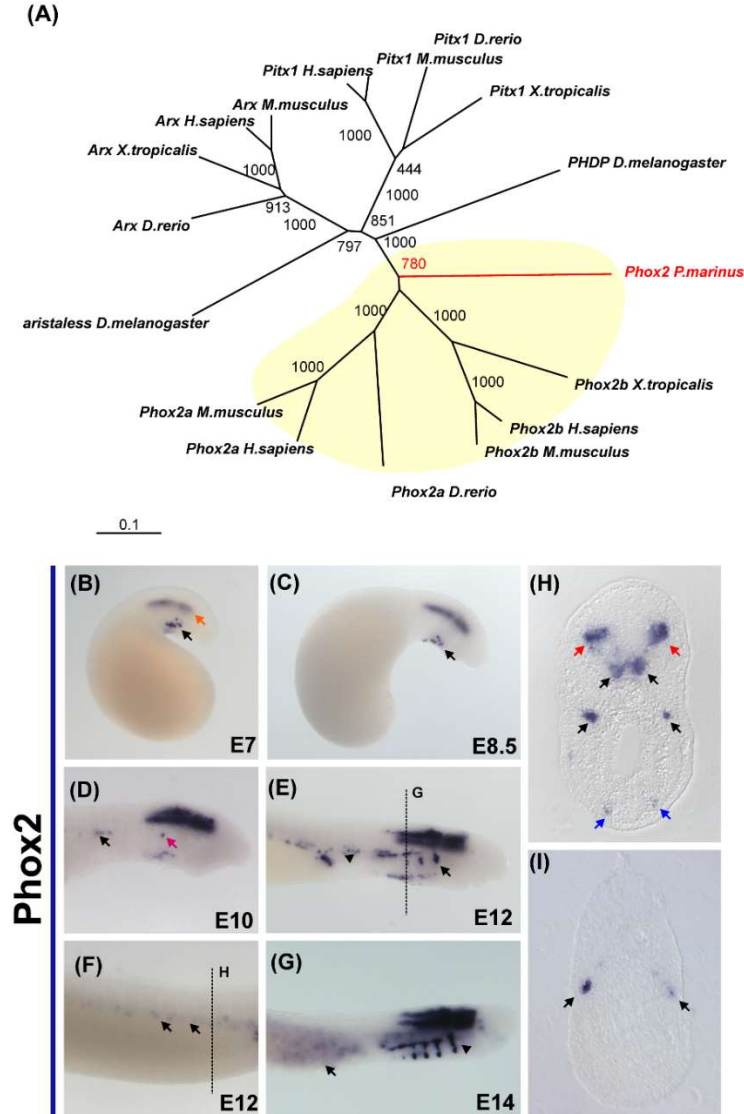
### **Author Contributions**

Conceived and designed the experiments: MSC TSS MBF. Performed the experiments: DH MSC BU JV MBF. Analyzed the data: DH MSC TSS MBF. Wrote the paper: DH MSC MBF.



**Figure 1. DiI labeling of lamprey neural crest cells reveals absence of sympathetic ganglia during embryonic development.**

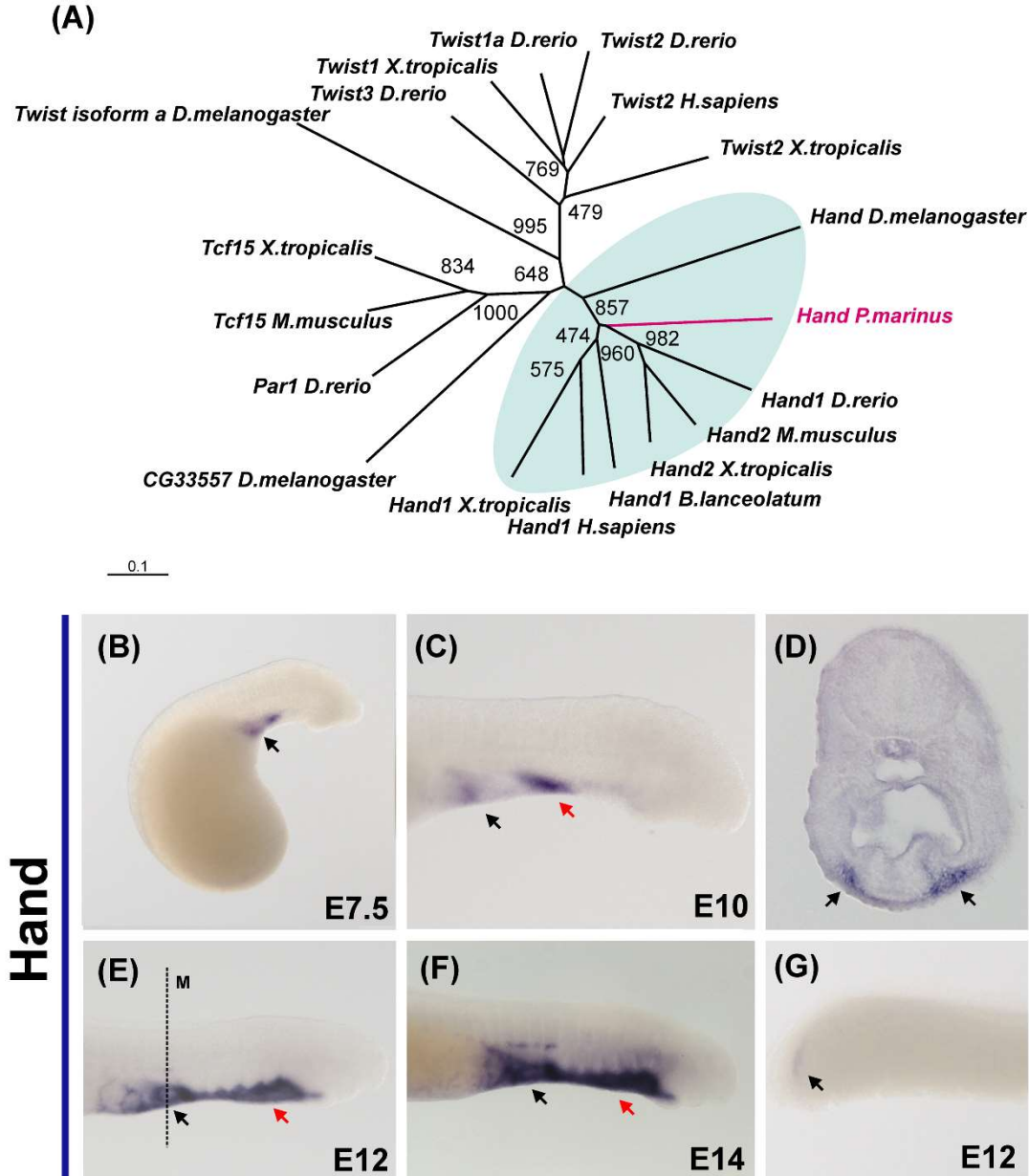
(A) Focal injections of DiI in the lamprey neural tube at day 5 result in labeling of migrating cephalic neural crest (arrow in B). However, focal injections into the posterior neural tube (C) fail to label trunk neural crest cells. D) Filling the lumen of the neural tube with DiI after cavitation produces labeled trunk neural crest cells in several neural crest derivatives (E). A section through an injected embryo (F) shows labeling of the dorsal root ganglia (DRG, arrow in G), the mesenchyme of the fin (H) and neurons surrounding the gut (F), but no structure that resembles sympathetic ganglia. Neurofilament staining (J) labels neural crest derivatives such as the DRG but also fails to reveal any sympathetic-like structures.



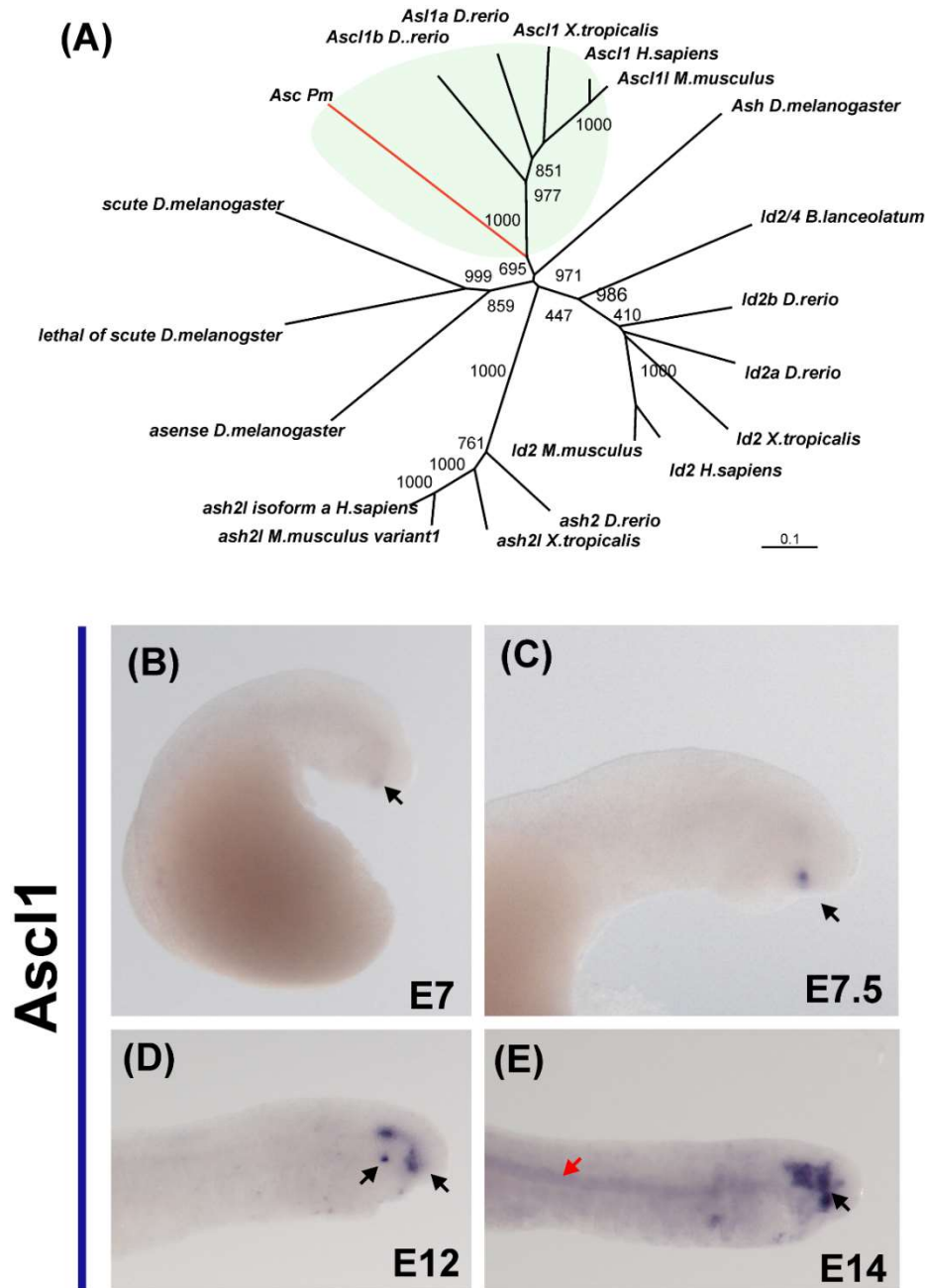
**Figure 2. Expression of the transcription factor Phox2 in embryos of *P. marinus*.**

(A) Phylogenetic analysis of a putative Phox2 fragment places it at the base of the gnathostome Phox2a and Phox2b gene families. (B) Expression of Phox2 is initially observed on the hindbrain (red arrow) and on a group of cells of the ventral mesenchyme (black arrow). (C) This expression pattern is maintained at day 8.5, as Phox2 positive cells migrate ventrally (black arrow). (D) At day 10, a stream of positive cells is observed above the heart and appears to be migrating posteriorly (black arrow). At this stage, Phox2 transcripts are first detected on the epibranchial ganglia (red arrow). (E) At day 21, the expression domain of Phox2 expands to include cranial (black arrow) and epibranchial ganglia (arrowhead). A section of this embryo (H) reveals staining in the motor neurons of the hindbrain, epibranchial ganglia and ventral mesenchyme (red, black and blue arrows, respectively). (F) At posterior axial levels, Phox2b expressing cells are observed adjacent to the yolk sac (section on I). (G) At later stages, a larger number of cells around the yolk start expressing Phox2 (black arrow), and there is strong expression in cranial nerves (arrowhead).





**Figure 3. Expression of the helix-loop-helix transcription factor Hand in lamprey embryos.** (A) Phylogenetic analysis suggests lamprey have one ortholog of both the dHand and eHand gnathostome genes. (B) Lamprey Hand expression is first observed in the cardiac field, and is conspicuous after 7 days of development. (B) At day 10, two domains of expression are clearly present: the heart (black arrow) and a part of the anterior mesenchyme (red arrow). (C) Expression in the cardiac ganglia (black arrow) is first detected at day 12; transverse sections reveal high abundance of transcript in the mesenchyme flanking the pharynx (black arrows on F). (E) Strong expression is observed in the ventral mesenchyme of the lamprey head (read arrow), as well as the heart at day 14 (black arrow). (G) Posterior expression appears to be restricted to the unsegmented mesoderm of the tail (black arrow).



**Figure 4. Expression of *Ascl1* during embryonic development of the lamprey.**

(A) Phylogenetic analysis places the putative lamprey *Ascl1* ortholog with gnathostome *Ascl1* genes. (B) Onset of *Ascl1* expression is apparent at day 7, when transcripts are detected in the anterior lip mesoderm. Shortly afterwards (C), faint expression is observed in the lens (arrow). (D) On day 12, lens expression is elevated (arrow), and staining on the hypophysis and trigeminal ganglia (red arrow) is also observed. (E) Finally, on day 14, *Ascl1* expression is observed in the notochord (red arrow).

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*Chapter 2 - 2*

**PROGRESSIVE REGIONALIZATION OF THE NEURAL CREST DURING  
VERTEBRATE EVOLUTION VIA ELABORATION OF GENE REGULATORY  
CIRCUITRY**

Benjamin Uy, Marcos Simoes-Costa, Daniel Koo and Marianne E. Bronner

**Abstract**

The neural crest of jawed vertebrates is divided into discrete subpopulations—cranial, vagal, trunk, and lumbosacral—distributed along the embryonic body axis with each following migratory pathways and forming a different set of derivatives. To gain insight into how these different neural crest territories may have arisen during vertebrate evolution, we analyzed the fate, behavior, and molecular identity of neural crest cells along the body axis of the sea lamprey (*Petromyzon marinus*), a basal jawless vertebrate. DiI fate-mapping revealed the presence of distinct cranial and trunk neural crest subpopulations, with a sharp transition occurring between them in the neural tube caudal to approximately somite 7. In contrast to jawed vertebrates, no intermediate population was apparent. We next performed comparative transcriptome analysis of lamprey cranial and trunk neural crest cells. The results revealed fewer cranial/trunk differences than observed in amniotes and several missing cranial crest-specific transcription factors. These results suggest that, in addition to the deeply conserved pan-vertebrate core neural crest gene regulatory network, there has been extensive elaboration of axial level specific regulatory circuits, likely contributing to the expansion of neural crest derived cell types during evolution of jawed vertebrates.



## Introduction

Evolution of vertebrates is intimately linked to the advent of the neural crest, a multipotent embryonic stem cell population. Neural crest cells give rise to many defining vertebrate characters, including the craniofacial skeleton, skull, and peripheral ganglia (Green et al., 2015). In fact, according to the “New Head” hypothesis, acquisition of the neural crest in vertebrates facilitated active predation, thus enabling elaboration of the brain (Gans and Northcutt, 1983). Neural crest cells arise from the dorsal midline of the central nervous system, but subsequently leave the neural tube and migrate extensively to various locations. Upon arrival at their destinations, they differentiate into a wide range of derivatives that range from neurons and glia of the peripheral nervous system to pigment cells as well as bone and cartilage of the face (Simões-Costa and Bronner, 2015).

Along the body axis of jawed vertebrates (gnathostomes), several populations of neural crest cells have been defined according to differences in their migratory pathways and types of derivatives into which they differentiate (Le Douarin, 1982; Simões-Costa et al., 2012; Simões-Costa and Bronner, 2015). Regionalization of the neural crest was crucial for the evolution of the vertebrate body plan, as each subpopulation follows distinct migration pathways and contributes to some unique derivatives. For example, in the head region, cranial neural crest cells contribute to cranial sensory ganglia as well as the craniofacial skeleton. In jawed vertebrates, cardiac/vagal neural crest cells arise from the neural tube just caudal to the ear and contribute to the septation of the outflow tract and heart, as well as enteric ganglia (both the submucosal and myenteric plexuses) of the gut. Just caudal to that, trunk neural crest cells form dorsal root ganglia, sympathetic ganglia, and adrenal chromaffin

cells. Finally, lumbosacral neural crest cells contribute to enteric glia of the rectum and portions of the posterior colon. Neural crest cells at all axial levels form melanocytes (Le Douarin 1982).

A pan-vertebrate neural crest (NC) gene regulatory network (GRN), invoking sequential deployment of signaling and transcriptional events, has been proposed to be responsible for formation of this unique cell type (Meulemans et al., 2004; Simoes-Costa and Bronner, 2015). Primarily studied at cranial levels, the NC GRN appears to be largely conserved across vertebrates (Sauka-Spengler et al., 2007), including the basal lamprey, a jawless vertebrate (agnathan). However, differences were noted in the position of key specifier genes, like cranial neural crest markers *Ets1* and *Twist*, which seem to be deployed later in the lamprey GRN than in other vertebrates (Sauka-Spengler et al., 2007). This suggests that there may be significant regulatory differences underlying the molecular machinery controlling neural crest identity in agnathans and gnathostomes.

Consistent with this, lamprey and other agnathans lack some neural crest derivatives emerging from various axial levels, *e.g.*, jaws at cranial levels, cardiac septum at cardiac/vagal levels, and sympathetic ganglia at trunk levels. This raises the intriguing possibility that differences in axial regionalization of the lamprey neural crest may contribute to the absence of these cell types. Furthermore, little is known about the evolution of neural crest subpopulations along the body axis. To tackle these questions, we have probed the behavior, derivatives, and molecular identity of the neural crest along the embryonic axis of the sea lamprey (*Petromyzon marinus*), a basal jawless vertebrate, compared with known data from gnathostomes.

Our results show that while lampreys possess a clear division between the cranial and trunk neural crest cells, they seem to lack an intermediate population that in jawed vertebrates would contribute to septation of the heart and the enteric nervous system. Furthermore, molecular analysis of lamprey cranial neural crest suggests that it expresses fewer cranial specific genes than observed in jawed vertebrates. Taken together, our data support a scenario in which the neural crest of lamprey was regionalized into cranial and trunk subpopulations, with the subsequent advent of the cardiac/vagal neural crest with emergence of gnathostomes. We postulate that elaboration of the molecular circuitry that controls the development of the cranial neural crest may have impacted neural crest morphogenesis, morphology of the vertebrate skull, and other derivatives during evolution of vertebrates.

## Results

### **Axial differences in the lamprey neural crest revealed by DiI fate-mapping**

In lamprey, the neural tube forms by cavitation, similar to teleosts, transforming from a neural rod into a tube with a lumen. At embryonic day (E)5 (Tahara 20), the neural rod elevates, neural crest precursors arise and the head begins to extend. Cavitation initiates around E6 (T21), at which time the neural crest primordia appear as bulges on the dorsal neural tube.

To characterize the neural crest subpopulations in the lamprey, we fate mapped the premigratory neural crest along the anteroposterior axis of the embryo. A single focal spot of DiI was introduced into the dorsal midline at different positions along the body axis at E5 to E5.5 (T19-T20) (Figure 1A-C). Labelled embryos were then observed at different stages of development to follow the patterns of neural crest migration and differentiation into various derivatives. Consistent with previous reports (McCauley and Bronner-Fraser 2003, Haming, Simoes-Costa et al. 2011, Modrell, Hockman et al. 2014), we found that the cranial crest contributed to the head mesenchyme and cranial ganglia (Figure 1D), while the trunk neural crest differentiated into neurons of the dorsal root ganglia and mesenchymal cells of the dorsal fin (Figure 1E).

Within the cranial region, DiI-labeling at the level of the forebrain (Figure S1A) generated labeled cells in the frontonasal process and in the trigeminal stream (Figure S1B). Labeling of the midbrain and rostral hindbrain produced labeled cells that invaded the maxillary and mandibular portions of the first arch (Figure S1C-D). These pathways match well with those reported previously after labeling lamprey embryos at E6 (McCauley and Bronner-Fraser, 2003) and also are similar to migratory pathways observed in gnathostomes

(Bronner-Fraser, 1993, Serbedzija 1992 et al., 1992, Selleck et al., 1993) (n = 32). DiI labeled cells possessed neurofilament-M labeled neuronal projections that were observed in the trigeminal ganglion, again similar to results in gnathostomes (Figure S1: E-H').

We next analyzed whether at later stages, DiI labeled cells contributed to heart or neurons of the gut, as they do in gnathostomes. Despite hundreds of focal injections into the neural tube adjacent to approximately somites 1-3, no labeled cells were observed in the vicinity of the heart or aortic vessels. For focal injections into the dorsal neural tube adjacent to the region of approximately somites 4-7, we observed DiI-labeled cells within the branchial arches, but in no cases were these observed to enter the heart or gut.

In order to label trunk neural crest populations, we performed focal DiI injections in the dorsal neural tube adjacent to somites 8-30 at E5-E6.5. This resulted in labeling a small number of migrating trunk neural crest cells. Embryos were sectioned at E14 through E21 to look at the distribution of DiI-labeled cells. At all stages examined, the results were similar. We noted that a small number of cells had migrated and/or delaminated from the neural tube to form the dorsal root ganglia (Figure 1B & D). In addition, mesenchymal cells were observed in the dorsal fin, similar to their distribution pattern in frog and fish embryos (Collazo et al., 1993; Smith et al., 1994).

Injections of DiI into the most caudal "sacral" portion of the neural tube at stages E5-E7 (n=23) (Figure S2 A & D) were indistinguishable from those injected into the trunk neural tube. Labeled cells were noted in the dorsal root ganglia and ectoderm of the tail fin (Figure S2 B, C, E, & F).

### **Partial conservation of cranial neural crest gene regulatory network in agnathans**

Our results suggest a clear distinction between the cranial and trunk subpopulations, with no obvious intervening population. Similarly, we failed to detect a “lumbosacral” neural crest population in lamprey. Thus, the ancestral state in stem vertebrates may be only to have distinct cranial and trunk neural crest populations. While our fate map analysis highlights important differences between the cranial and trunk neural crest cells, previous studies have shown that the lamprey lacks expression of cranial neural crest genes, such as *Ets1* and *Twist* (Sauka-Spengler et al., 2008), in the migratory population. This raises the intriguing possibility that important changes in the molecular mechanisms controlling cranial neural crest development may have accompanied early evolution of jawed vertebrates. To explore this hypothesis, we surveyed the conservation of the cranial neural crest gene regulatory network in the lamprey.

Recently, we have described a cranial-specific gene regulatory circuit that is important for cranial neural crest formation and differentiation in amniotes. This circuit includes *early* transcription factors like *Brn3c*, *Lhx5*, and *Dmbx1*, which activate the expression of *intermediate* and *late* regulators *Ets1*, *Tfap2b*, and *Sox8* in the cranial neural crest (Figure 2A). Other genes, such as *Id2* (Martinsen and Bronner-Fraser 1998), *Twist* (Gitelman 1997), and *Axud1* (Simoes-Costa et al., 2015) have also been shown to be enriched in the cranial neural crest and play important roles during its development. To verify if this cranial neural crest “regulatory state” is conserved amongst all vertebrates, we took a candidate approach to analyze the expression patterns of the orthologs of these genes in lamprey embryos at different stages of development. We found that while *Id2* (*Id*), *Sox8* (*SoxE1*), and *Tfap2b* (*Tfap2c*) were robustly expressed in the lamprey premigratory and

migratory cranial neural crest (Figure 2B), a large number of cranial regulators appear to be absent from this cell population. In contrast to what has been described in amniotes, we were unable to detect genes like *Brn3c*, *Lhx5*, *Dmbx1* in the cranial neural crest or anterior neural folds (Figure 2B). Although present in the genome, lamprey cranial neural crest cells appear to lack a large part of the cranial-specific regulatory circuit (Figure 2C), pointing to a high degree of regulatory divergence between the regulatory states of jawed and jawless neural crest.

The analysis of expression patterns of cranial genes in agnathans can provide important clues on how this regulatory sub-circuit evolved. Intriguingly, we found that genes from the cranial circuit that are absent from the early neural crest are co-expressed at later stages in the branchial arches. As previously described by Sauka-Spengler and colleagues (2008), *Ets1* is transcribed by the differentiating post migratory branchial arches neural crest, cranial placodes, and dorsal root ganglia. Our analysis shows that transcripts of both *Lhx5* and *Dmbx1* are also present in the post migratory branchial arches neural crest (Figure 2D), raising the possibility that terminal regulatory circuits might have been re-deployed to play an earlier role in neural crest specification in gnathostomes. According to this scenario, genes involved in the differentiation of the neural crest in agnathans may have been co-opted by the specification program in gnathostomes (Green et al., 2015), possibly endowing the cranial neural crest with novel morphogenetic features.

### **Transcriptome analysis of the lamprey cranial neural crest**

The candidate gene approach described above suggests that extensive changes occurred in the neural crest regulatory state between jawless and jawed vertebrates during

vertebrate evolution. To further investigate this hypothesis, we conducted an unbiased analysis of the lamprey cranial neural crest through comparative transcriptome analysis of cranial and trunk neural crest subpopulations.

To obtain premigratory neural crest populations, we microdissected segments of cranial and trunk dorsal neural tubes at stages E6.5 and E8, respectively (Figure 3A-B), and extracted total RNA from these samples for transcriptome analysis. In this manner, we identified 811 genes significantly enriched in lamprey cranial neural crest (Figure 3C) when compared to the trunk. Consistent with our previous *in situ* hybridization analysis (Figure 2B), cranial genes in the cranial dataset included transcription factors *Id2* (Id), *Sox8* (SoxE1), and *Tfap2b* (Tfap2c). This indicated that our dataset was suitable for identification of cranial specific genes in the lamprey, and accordingly we employed it to conduct two types of analysis. First, we took a broader look at the conservation of vertebrate cranial neural crest genes in the lamprey by comparing our dataset with an amniote transcriptome. Second, we investigated the possibility that the lamprey has a cranial neural crest regulatory program that is distinct from those characterized in jawed vertebrates.

Taking advantage of a recent study in the chick that identified 160 genes enriched in the cranial neural crest when compared to the trunk (Simoes-Costa and Bronner, under review), we compared our lamprey cranial neural crest transcriptome with this chicken dataset. Interestingly, the results show that only 9.7% of chicken cranial neural crest genes are similarly enriched in lamprey compared with trunk neural crest (Figure 3D). The vast majority of the genes 70.8% showed no significant enrichment in the cranial versus trunk populations. In contrast, 19.4% were enriched in the trunk. This analysis supports our



previous results showing low conservation of the cranial neural crest regulatory program in agnathans compared with gnathostomes.

Finally, we investigated the hypothesis that the lamprey might have a different set of cranial regulators involved in neural crest formation. To accomplish this, we examined the expression patterns of the 20 genes that were most enriched in our cranial neural crest dataset. Included in this group are Sox8 (SoxE1), Tfap2b (Tfap2c) (Figure 3E-F), and Id2 (Id), which are strongly expressed in the lamprey neural crest. However, we found that the other genes in that category, such as Tdo2b, Rbp4l, Vill, Dpy19l3, Cdo1b, and Sspo, were indeed strongly restricted to the cranial regions, but absent from the neural crest (Figure 3G-L), being expressed in the central nervous system. Thus, we were unable to identify cranial neural crest genes that were lamprey specific. Taken together, these results support the hypothesis that agnathans possess a simpler cranial neural crest gene regulatory network, which might have important implications for the evolution of the neural crest subpopulations.

## **Discussion**

The neural crest is a cell type that is present in both jawless fish (agnathans) and jawed vertebrates (gnathostomes). Agnathans, like the lamprey, are missing some critical neural crest derivatives like jaws, cardiac septum, and sympathoadrenal cells, which in gnathostomes arise from “cranial”, “cardiac/vagal”, and “trunk” neural crest populations, respectively (Sauka-Spengler et al., 2007). To gain insight into whether differences in neural crest migratory pathways may have contributed to differences between agnathans and gnathostomes, we performed a detailed fate map of neural crest migratory pathways along

the rostrocaudal body axis. To better understand how these subpopulations may have evolved, we also scrutinized the regulatory network of the lamprey cranial neural crest.

Our results confirm that cranial neural crest migratory pathways are similar to those observed in jawed vertebrates despite the lack of jaws. In contrast, posterior cranial neural crest cells arising from post-otic levels migrate ventrally and, upon reaching the branchial arches, move rostrally and caudally to occupy the mesenchyme of all arches. However, in contrast to jawed vertebrates, they fail to contribute to enter the heart/outflow tract or gut.

Our results suggest that the patterns of neural crest migration may be quite different in lamprey, a representative of basal vertebrates, from that in gnathostomes, at least at post-otic levels. In all vertebrates examined, including lamprey, the cranial neural crest cells arising rostral to rhombomere 3 migrate as a uniform population expands like a cobra's hood to populate the frontonasal process and first branchial arch, which will form the upper and lower jaw of vertebrates and surround the mouth in lamprey. These pathways appear to be remarkably conserved, suggesting that they are subject to strong evolutionary constraint. Caudal to the presumptive ear, however, differences were noted between posterior cranial neural crest cells in lamprey versus other vertebrates. Whereas in fish, frog, chick, and mouse, neural crest cells from the caudal hindbrain migrate directly into the branchial arches either above or through the adjacent somites, all lamprey caudal hindbrain neural crest cells, regardless of whether they arise adjacent to somite 1 or somite 7, appear to be funneled into a single migratory stream above the branchial arches. They proceed to migrate both rostrally and caudally through the mesenchyme, spreading evenly throughout all the branchial arches. Contrasting with observations in gnathostomes, they do not migrate toward the heart or gut.

Focal injections of DiI into the neural tube caudal to the somite 7 level are only able to minimally label the trunk neural crest. In embryos assayed between E16 and E30, DiI labeled cells were observed in the mesenchyme of the fin, dorsal root ganglia, and along spinal nerves, and occasionally in the typhlosole, but not in ventral locations corresponding to sympathetic ganglion formation. In general, the emigration of trunk neural crest cells from the neural tube occurs much later, after cavitation of the neural tube. These results suggest that, in contrast to the pre-otic neural crest in which migration pathways are highly conserved and perhaps subject to strong evolutionary constraint, there are marked differences at post-otic levels. This correlates with a lack of several critical post-otic neural crest derivatives like the cardiac septum, adrenal medulla, and sympathetic ganglia.

As a vertebrate innovation, the neural crest is present in all vertebrates examined to date including agnathans. In contrast, basal chordates like amphioxus completely lack neural crest cells. It has been proposed that urochordates may have some neural crest-like cells that have migratory properties and can form melanocytes (Jeffery et al., 2004). For example, it was shown that ectopic expression of *Twist* in a cephalic melanocyte lineage could cause this cell to become migratory, suggesting that it may represent a proto-neural crest cells (Abitua et al., 2012). Recently, a population of neurons, derived from precursors at the neural plate border that migrate along the paraxial mesoderm, appear to contribute to a type of sensory neuron in *Ciona* (Stofli et al., 2015), and thus possess both the ability to form peripheral neurons and to migrate, two important characteristics of the neural crest. However, only vertebrates have bona fide neural crest cells that form within the neural tube, migrate extensively and give rise to a multitude of derivatives.

Consistent with evolutionary expansion of neural crest cells in the vertebrate lineage,

our molecular analysis of the cranial neural crest reveals surprising differences in lamprey compared with gnathostome counterparts. These differences may help explain some of the evolutionary novelty that arose in the transition from jawless to jawed vertebrates. Our results show deep conservation of a few transcription factors in the lamprey cranial neural crest. SoxEs, Tfap2, and Id may be the rudiment of a larger, more complex gene regulatory network that was expanded during early vertebrate evolution with the incorporation of novel players (LMO4a, CSRNP, Brn3, Lhx5, Dmbx1, Ets1, Twist, and LZTS1) (Simoes-Costa and Bronner, under review). Our observation also shows that some of these “novel” genes are co-expressed at later stages of neural crest formation, consistent with the possibility that the elaboration of the cranial GRN might have taken place through cooption of parts of differentiation programs to earlier portions of the network.

The differences we observe in axial-specific genes contrasts with the deep conservation of the pan-neural neural crest program (Sauka-spengler et al., 2007). Thus, the pan-neural crest program was likely the ancestral molecular recipe to make neural crest, with the subsequent elaboration of the axial level specific regulatory programs conferring important differences in developmental potential to neural crest cells along the body axis. Given that lampreys lack several key neural crest derivatives, we postulate that the absence of these derivatives may be due gene regulatory differences associated with axial-level specific regulatory programs.

Taken together, our results suggest the following scenario to explain the evolution of the neural crest subpopulations (Figure 4). We postulate that the split of the neural crest into cranial and trunk subpopulations occurred early in vertebrate evolution, perhaps in the ancestral vertebrate such that the regionalization observed in the lamprey neural crest reflects

this ancestral state (although we cannot rule out that agnathans lost neural crest subpopulations during the course of evolution). The relative scarcity of cranial factors in the lamprey cranial neural crest might suggest that the GRN underlying this cell population has undergone extensive elaboration from a regulatory standpoint. Thus, we propose that regionalization of the neural crest, with both the emergence of the new subpopulations and the expansion of the cranial GRN, played a crucial part in vertebrate evolution, culminating in the rise of the gnathostomes.

## **Experimental Procedures:**

### ***DiI labeling in lamprey embryos***

Embryonic day(E) 5 -7 day old embryos were manually dechorionated in 0.1xMMR and placed into agarose-coated Petri dishes. DiI solution [(Cell Tracker-CM-DiI Invitrogen) 0.5µg/µl, prepared in 0.3M sucrose] was injected into the respective regions along the rostrocaudal axis. Embryos were raised in 18mm agarose coated petri dishes containing 0.1xMMR at 18°C and the migration of DiI labeled cells were analyzed every day by fluorescence microscopy. Once the embryos had reached the desired stage, they were fixed using 4% paraformaldehyde in PBS at room temperature for 1hr or 16 hours at 4°C.

### ***Embedding, sectioning, and immunostaining of lamprey embryos***

Post fixation (1 hour 4% paraformaldehyde at room temperature), lamprey embryos were washed with PBST 3 times for 15min. Subsequently, they were incubated in 5% sucrose in PBS for 15 mins and 15% sucrose in PBS for 16-24 hours at 4 degrees,

in 7.5% gelatin and 15% sucrose for 12 hours at 37°C. Embryos were placed into 7.5% gelatin and 15% sucrose, positioned, and sectioned at 12-20 µm with a *Microm* HM550 *cryostat*.

Immunostaining of lamprey embryos was performed as previously described (Nikitina et al, 2009). Anti-Neurofilament (NF-M, Mouse IgG2a, Invitrogen) antibody was used 1:200 and anti-type II collagen antibody supernatant (Developmental Studies Hybridoma Bank, II-II6B3, Mouse IgG1) was used at 1:10. Secondary antibodies were used at 1:500. Slides were mounted with Fluoromount-G (Southernbiotech).

#### ***In situ hybridization and histology.***

Whole-mount *in situ* hybridization of lamprey embryos was performed using digoxigenin RNA probes according to Wilkinson (Xu et al., 1990), with following modifications: Prior to Proteinase K step, embryos equilibrated in the bleaching solution (0.5X SSC, 5% formamide, 10% H<sub>2</sub>O<sub>2</sub>), were exposed to direct light using light box for 10-15 minutes. The concentration and the length of Proteinase K treatment (~20 µg/ml, 10 minutes) was the same for embryos of all stages. Hybridization and subsequent washes were carried out at 70°C in hybridization solution containing 50% formamide; 1.3X SSC; 5mM EDTA pH8.0; 200 µg/ml yeast tRNA; 100 µg/ml heparin; 0.2% Tween-20 and 0.5% Chaps. The hybridization signal was detected using BM Purple substrate (Roche, Indianapolis, IN) for early stage embryos (E3-E10) or NBT/BCIP (Roche, Indianapolis, IN) for later stages.

### ***Isolation and Cloning of the PCR product***

Orthologs were identified by bioinformatic survey of the lamprey genomic sequences from the ensembl database. Total RNA was extracted from embryos using the Ambion: RNAqueous kit. PCR was performed with Roche:GC-Rich PCR system according to protocol with a 50 C annealing temperature. LHX5 and DMBX1b were synthesized from GenScripts based on their ensembl sequences.

Extraction of the PCR product was conducted corresponding to Qiagen:QIAquick Gel Extraction Kit and cloned with Invitrogen: TOPO TA Cloning. The clones were selected against the metabolism of X-gal and the production of  $\beta$ -galactosidase purified following the QIAprep spin miniprep kit and sent for sequencing. (Laragen Inc., Culver City, CA)

### ***RNA-Seq and Transcriptome analysis***

Biological samples were dissected from the cranial dorsal neural tubes of E6.5 and trunk neural tube of E8.5 lamprey embryos. Total RNA was extracted from embryos using the Ambion: RNAqueous kit. RNA-Seq was performed at the Millard and Muriel Jacobs Genetics and Genomics Laboratory (California Institute of Technology, Pasadena, CA) at 35 million reads on 2 biological replicates and 1 whole embryo control for both the E6.5 cranial and E8.5 trunk neural tube samples.

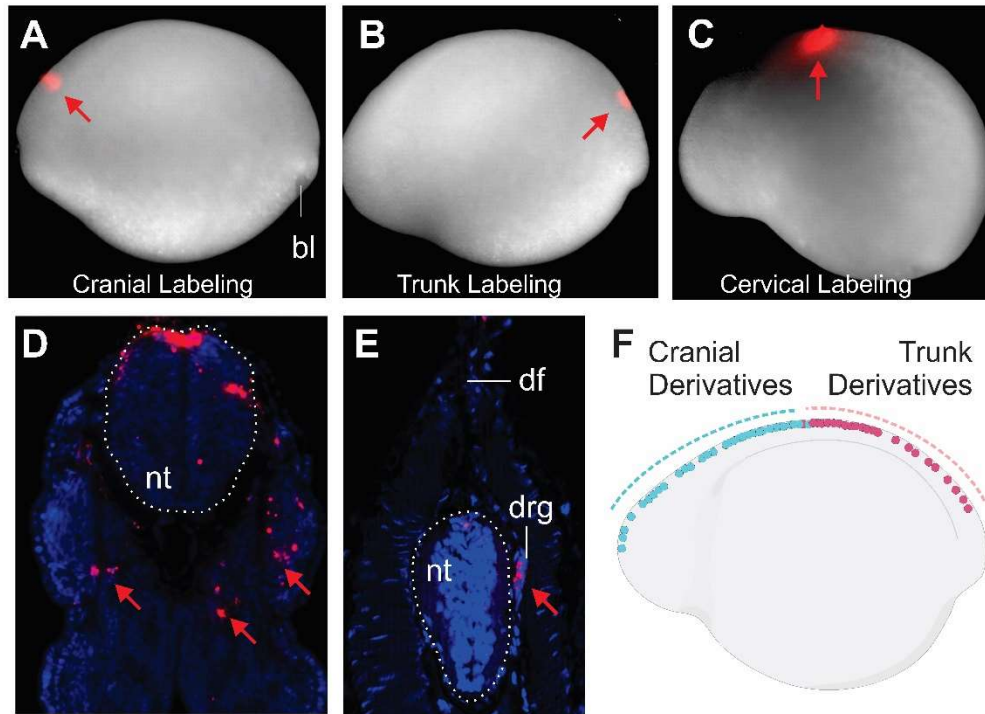
Sequencing libraries were built according to Illumina Standard Protocols. SR50 sequencing was performed in a HiSeq Illumina machine. Sequence reads were aligned to the *Petromyzon marinus* genome with Bowtie (version 0.12.7) and TopHat (version

1.3.1.OSX\_x86\_64). DE-seq was used to calculate gene expression levels and identify differentially expressed transcripts.

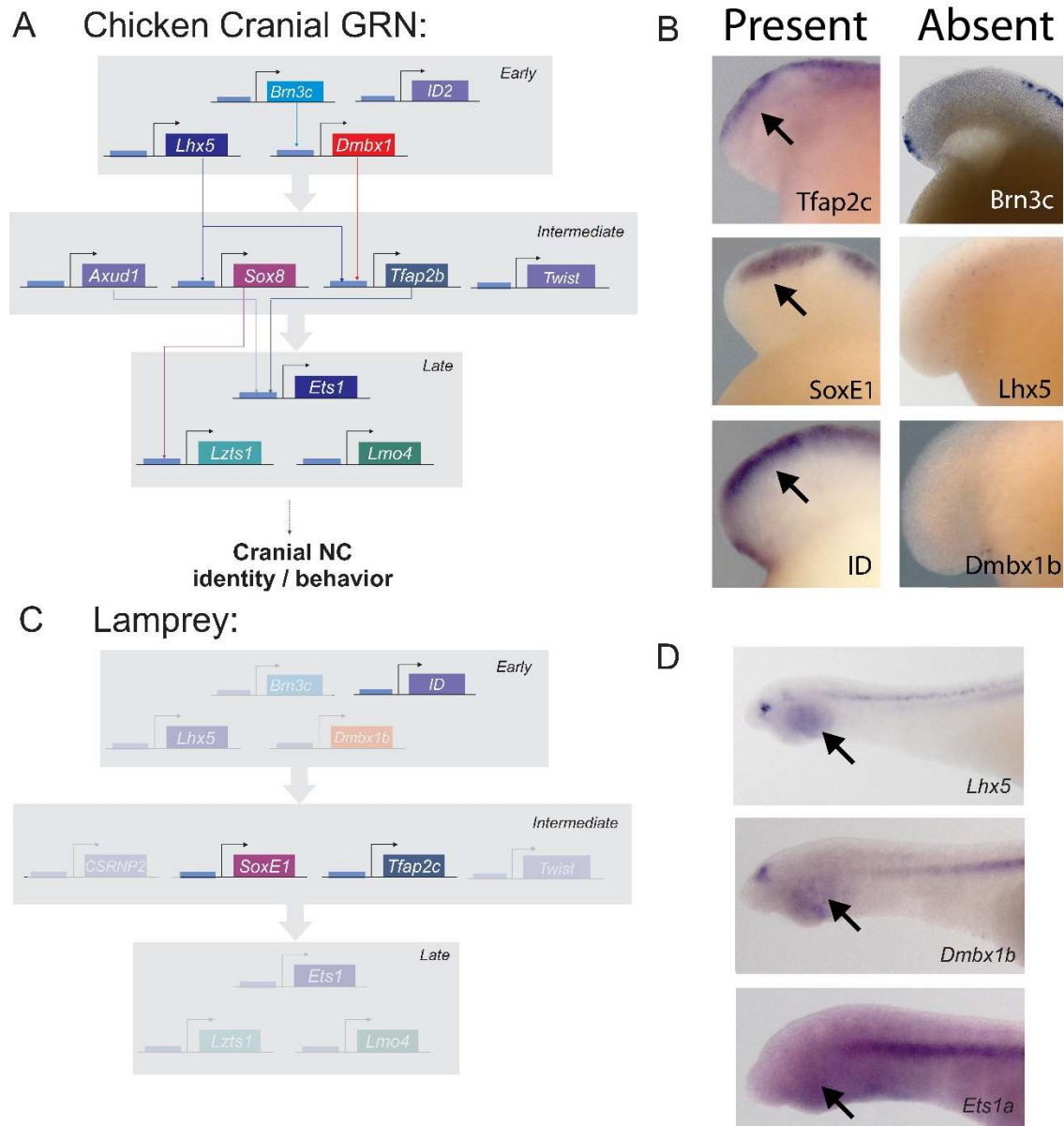
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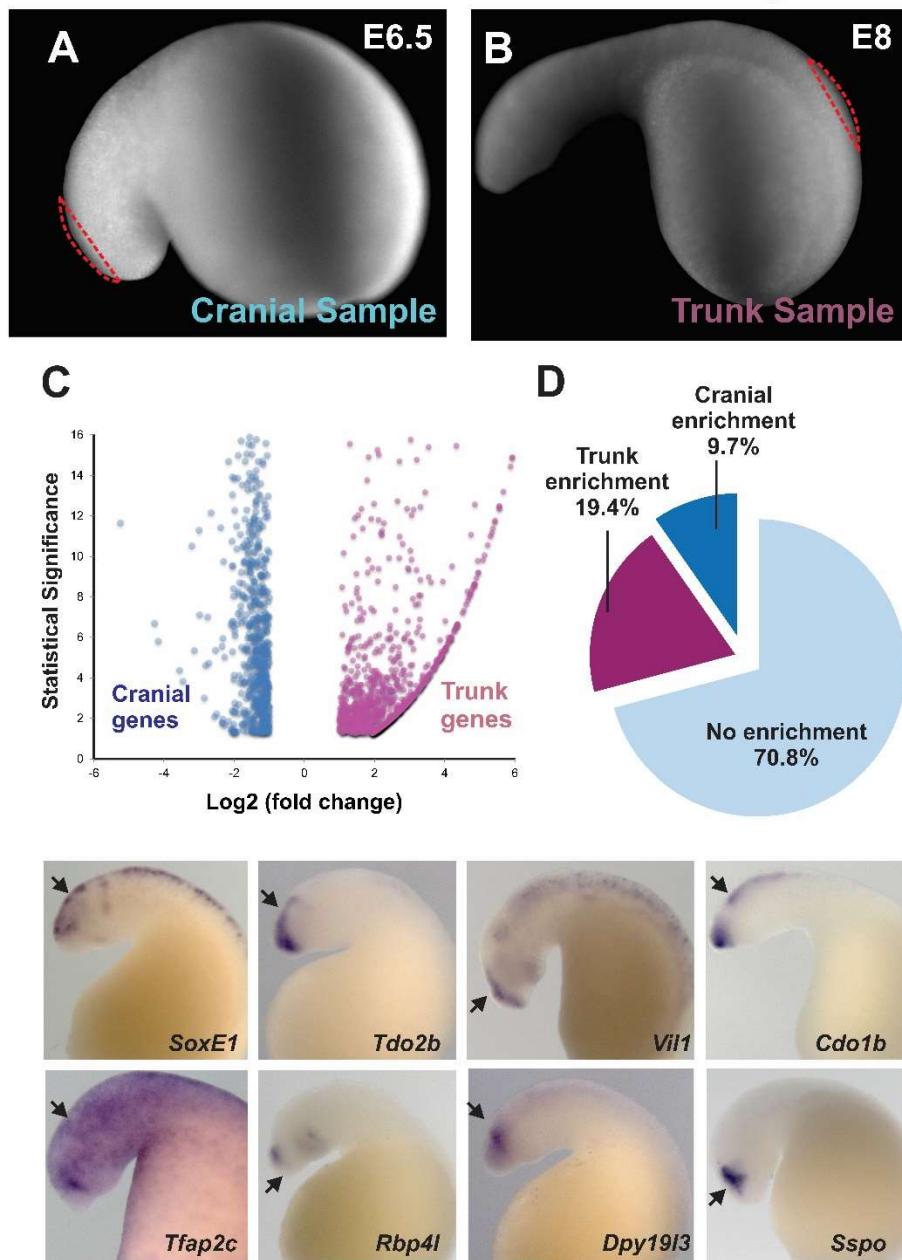


**Figure 1: DiI-labeling along the anterior-posterior body axis reveals distinct populations of cranial and trunk neural crest cells.** A-C: Initial injection points (time=0) at E5.5-E6. A) Example of a cranial injection that, at later stages, contributes to trigeminal ganglia and branchial arches. B) Sample trunk injection which at later stages contributes to dorsal root ganglia (drg). C) Sample cervical injection which at later stages contributes cells to branchial arches. D) Transverse cross section of a E16 embryo showing DiI along the trigeminal ganglia after injection as in (A). Red: DiI; Blue: DAPI. E) Transverse cross section of E20 embryo with DiI within the dorsal root ganglion (drg). Red: DiI; Blue: DAPI. F) Summary fate map of initial injections data points along the anterior-posterior axis with their respective fates. Each dot represents an embryo injected at the respective position. Cyan: Cranial fates (n=32) (Trigeminal ganglia and branchial arch contributions). Magenta: Trunk fates (n=23) (drg and fin mesenchyme contributions); bl:blastopore; nt: neural tube; df: dorsal fin; drg:dorsal root ganglia. Arrows indicate DiI-labeled cells.



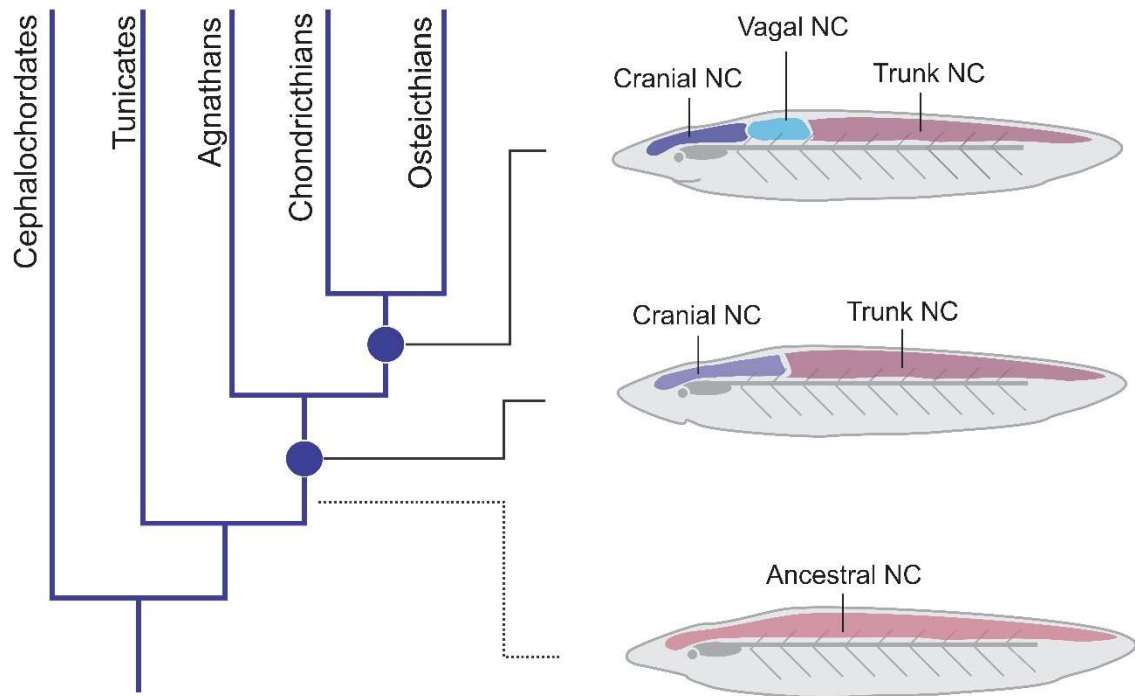
**Figure 2: Comparison of chick cranial-specific gene regulatory circuit with that of lamprey.**

a) Diagram of the chicken cranial neural crest specific gene circuit described by Simoes-Costa et al. (2016). b) *In situ* hybridization analysis of lamprey orthologs of these genes shows conserved expression of *Tfap2c*, *SoxE1*, and *Id* in the premigratory neural crest; however, *Brn3*, *Lhx5*, and *Dmbx1b* were not expressed in the neural crest. In addition, *Twist*, *Ets1a/b*, *CSRNP2* are also absent in premigratory NC (data not shown). c) Model of lamprey orthologs cranial crest GRN with *Id*, *SoxE1*, and *Tfap2c*. d) *In situ* hybridization of E9 embryos for *Lhx5*, *Dmbx1b*, and *Ets1a* demonstrates that they are expressed later in the branchial arches.

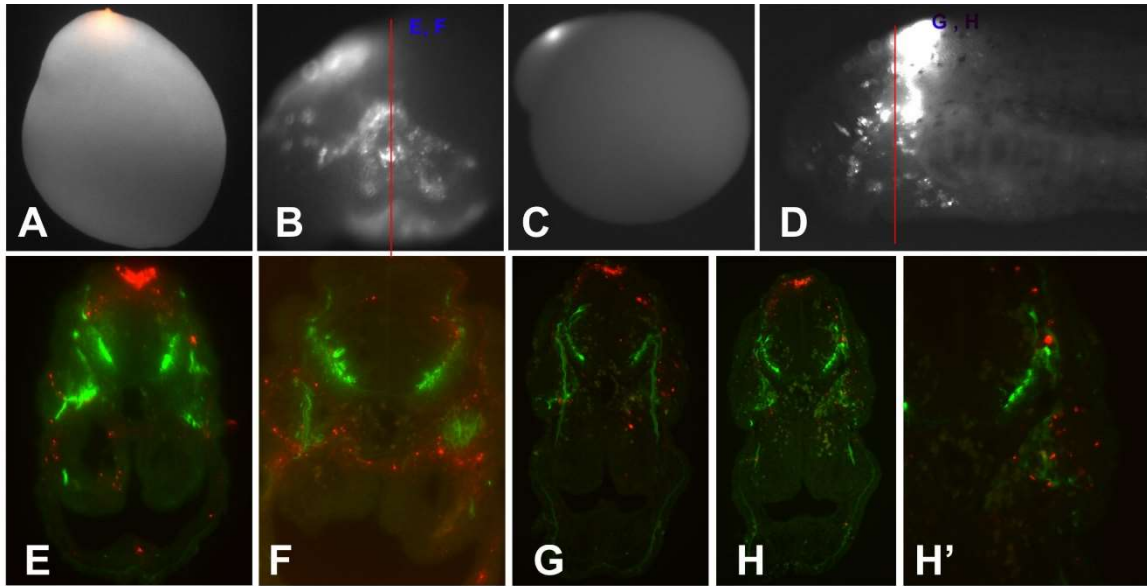


**Figure 3: Transcriptome analysis comparing lamprey cranial and trunk neural crest.**

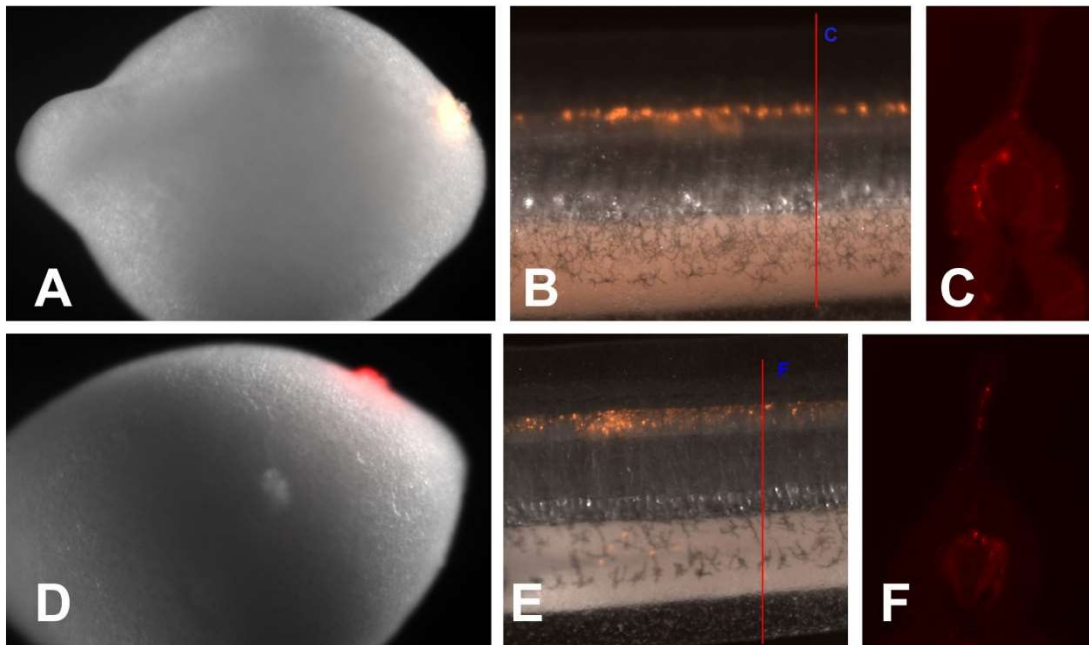
A) Cranial neural crest samples of the dorsal neural tube taken at E6.5 (red outline). B) Trunk neural crest samples of the dorsal neural tube taken at E8 (red outline). C) Scatter plot of  $-\log$  adjusted p-value vs  $\log_2$  fold change (y threshold:  $P_{adj} < 0.05$ ), x threshold:  $\log_2$  fold change = 1). (Cyan) 811 Cranial Enriched Genes. (Magenta) 1000 trunk enriched genes. D) Pie chart comparing lamprey cranial ortho enrichment to chick cranial enrichment. Cyan: 9.7% Cranial Enrichment (7/72) ( $>1.0$   $\log_2$  fold change), Magenta: 19.4% Trunk enrichment (14/72) ( $<-1.0$   $\log_2$  fold change), & Blue: 70.8% No significant enrichment (51/72). E-L) *In situ* hybridization expression of E8 embryos E) *SoxE1*, F) *Tfap2c*, G) *Tdo2b*, H) *Rbp4l*, I) *Vil1*, J) *Dpy19l3*, K) *Cdo1b*, & L) *Sspo*.



**Figure 4: Proposed model for evolution of neural crest subpopulations.** Phylogenetic tree (arms not drawn to scale) of various groups postulating that an earlier common ancestor possessed an ancestral neural crest population that may have been similar to the trunk neural crest. This progressed to the emergence of two subpopulations—cranial and trunk neural crest—in agnathans prior to the separation of jawed and jawless vertebrates. After the separation, jawed vertebrates further developed an intermediate vagal population and caudal lumbosacral population.



**Supplemental 1: Cranial Neural Crest contribute to the trigeminal ganglia.** (A) Initial injection point at E5 and (B) at final stage of E16. (C) Initial injection point of an E5.5 embryo and (D) final stage E16. (E-H') At E16, DiI (red) labeling is observed in the trigeminal ganglia and transverse sections show that some cells express Neurofilament-M (Green).



**Supplemental figure 2: Trunk neural crest give rise to dorsal root ganglia and fin mesenchyme.** A,D) Initial labeling of the neural tube at E6 and (B,E). Final stage of analysis at E20. (C,F) Transverse sections showing DiI in the dorsal root ganglia.

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*Chapter 3***ANCIENT EVOLUTIONARY ORIGIN OF THE VERTEBRATE  
ENTERIC NERVOUS SYSTEM FROM SCHWANN CELL  
PRECURSORS**

Benjamin Uy\*, Stephen Green\*, and Marianne E. Bronner



**Abstract**

The enteric nervous system (ENS) of jawed vertebrates arises from vagal neural crest cells that migrate to the foregut and subsequently colonize and innervate the entire gastrointestinal tract. To gain insight into its evolutionary origin, we examined ENS development in the basal jawless vertebrate, the sea lamprey. Surprisingly, we found no evidence for the existence of a vagal neural crest population in the lamprey. Rather, *DiI* labeling showed that late-migrating cells, including those originating from the trunk neural tube and associated with nerve fibers, differentiated into neurons within the gut wall and typhlosole. We hypothesize that these trunk derived neural crest cells are homologous to Schwann cell precursors (SCPs) that have recently been shown to populate post-embryonic parasympathetic ganglia<sup>1,2</sup>, including enteric ganglia<sup>3</sup> in mammalian embryos. Our results suggest that neural crest derived SCPs made an important contribution to the ancient ENS of early jawless vertebrates, a role that was largely subsumed by vagal neural crest cells upon emergence of gnathostomes.

The enteric nervous system is composed of thousands of interconnected ganglia embedded within the walls of the gut<sup>4,5</sup>, making it the most complex portion of the peripheral nervous system in amniotes. The ENS innervates the entire gastrointestinal tract to regulate muscle contraction, water balance, and gut secretions of jawed vertebrates. Classical transplantation experiments have demonstrated that the neurons and glia of the gut are largely derived from the “vagal” population of neural crest cells that arise within the post-otic portion of the hindbrain<sup>6,7</sup>. These cells emigrate from the hindbrain, enter the foregut, and undergo the longest migration of any embryonic cell type to populate the entire length of the gut from foregut to hindgut.

The sea lamprey, *Petromyzon marinus*, is a jawless (agnathan) vertebrate, and a experimentally tractable representative of the cyclostomes, the sister group to all other (jawed) vertebrates, making lamprey an important model for identifying traits common throughout vertebrates. Lampreys possess migrating neural crest cells that give rise to many neural crest derivatives found in gnathostomes, including pigment cells, cartilage, sensory neurons, and glia, but they lack other neural crest-derived cell types that are present in gnathostomes like jaws and sympathetic chain ganglia<sup>8,9</sup>. Given that lamprey embryos lack sympathetic ganglia, we sought to examine other components of the autonomic nervous system, with focus on the enteric nervous system. Adult lamprey have a simple ENS that includes ganglionated plexuses of serotonin (5-HT) producing cells<sup>10</sup>, as well as a smaller number of catecholamine-containing neurons. However, the developmental origin of these enteric neurons is unknown.

As a first step in analysis of lamprey ENS development, we examined the time course of appearance of neurons along the developing embryonic gut using *in situ* hybridization.

*Phox2b* is expressed in enteric neurons in many jawed vertebrates<sup>8</sup>. Expression of lamprey *Phox2b* is detectable along the gut from early stages [Tahara 25<sup>8</sup>(T25)] but it is unclear whether these cells will become enteric neurons. By stage T28 (E20), *Phox2b* expressing cells are associated with a depression in the gut which will become the typhlosole, a hematopoietic tissue that is associated with elements of the enteric nervous system (Fig. 1A-B). Differentiated neurons were first observed within the gut wall at approximately T28 or embryonic (E) day 20 (Fig. 1C-D). At this time point, we noted serotonergic neurons in more rostral portions of the gut. With time, the numbers increased, and 5-HT<sup>+</sup> neurons were noted progressively posteriorly, with particularly high cell numbers in the cloacal region, as reported previously<sup>11</sup>. By E30, there are approximately 80-100 neurons along the gut, in association with the typhlosole and vagus nerve (Fig. 1E). Interestingly, differentiating neurons were often associated with nerve processes emanating ventrally from the spinal cord and along the vagus nerve that projects posteriorly along the gut (Fig. 1D). In addition to 5-HT<sup>+</sup> neurons, we also noted the presence of 5-HT<sup>+</sup> non-neuronal columnar cells that may represent enterochromaffin cells (SupFig. 1A-B).

We next sought to determine the embryological origin of the neurons in the gut by performing lineage labeling with the lipophilic dye, DiI<sup>1,2</sup>. In chicken, the vagal neural crest cells that contribute to the ENS arise from the hindbrain neural tube adjacent to somites 1-7<sup>6</sup>. After exiting the neural tube, they migrate ventrally, invade the foregut, and then undergo a collective cell migration along the rostrocaudal extent of the gut. To test whether lamprey possess a homologous cell population, we performed focal injections of DiI into the dorsal portion of the caudal hindbrain of T20 (E6) embryos, approximately adjacent to lamprey somites 1 through 8, region corresponding to the site of origin of vagal neural crest cells in

gnathostomes. Regardless of the exact injection site, dye labeled cells spread within the hindbrain and appeared to leave the neural tube at a single stream directly above the forming branchial arches. From this site, they progressed ventrally and then turned caudally to populate all of the branchial arches (SupFig. 2A-C), similar to previously reported migration patterns of cranial neural crest cells<sup>13,14</sup>. However, despite many focal injections throughout the caudal hindbrain, we failed to find evidence of DiI-labeled cells entering the gut suggesting a lack of vagal crest in the lamprey. We next looked for expression of the vagal neural crest marker, *ret*, in lamprey, since it is required for vagal neural crest development in gnathostomes<sup>8</sup>. To this end, we cloned a lamprey *ret* homolog and examined its expression pattern by *in situ* hybridization. Results show that lamprey *ret* is expressed in many parts of the embryo, including the typhlosole, but we did not observe expression in migrating neural crest cells (SupFig. 2 G-J), suggesting that enteric neurons might arise from a different cellular source.

In contrast to the vagal neural crest cells in gnathostome, migrating trunk neural crest cells normally fail to invade the immediately underlying gut due to the presence of repulsive signals like Slit that block their entry<sup>15</sup>. Recently, however, a secondary contribution to the mammalian ENS has been uncovered that comes not from the vagal neural crest but rather from trunk neural crest-derived Schwann cell precursors (SCPs) associated with extrinsic nerves that contribute postnatally to calretinin-containing neurons of the mammalian gut<sup>3</sup>. Intriguingly, this population persists in *ret* null mice<sup>3</sup>. To examine the possibility that a homologous population may exist in lamprey, we asked whether cells emerging from the trunk neural tube might contribute to neurons of the gut. To test this possibility, we performed both focal DiI injections in the dorsal trunk neural tube as well as luminal

injections following its cavitation at T22-T23 (~E8), thus labeling the entire neural tube as well as presumptive neural crest cells prior to their emigration. By T25 (E11-12), trunk DiI neural tube fills label neural crest derived cells in dorsal root ganglia and mesenchyme cells of the fin<sup>8</sup>. Interestingly, at later stages, labeling led to the presence of DiI-labeled cell bodies closely associated with nerve processes above the gut, as evidenced by staining of nerves with antibody to acetylated tubulin (Fig. 2A-C). These individual DiI-labeled cells were visible as early as E20 and persisted until the latest stages examined (E30), by which time the yolk had cleared in more anterior trunk regions, facilitating imaging of the gut. After trunk neural tube labeling, we noted DiI-positive cells in the vicinity of the gut in 47 embryos.

To establish whether the DiI-labeled cells differentiated into enteric neurons, embryos were sectioned and stained with antibodies to serotonin and acetylated tubulin as a mature neuronal marker. The results, based on examination of transverse sections through 25 representative embryos in which we quantitated cells that were both acetylated tubulin and serotonin-positive at E30 to E35, are summarized in Table 1. We noted numerous DiI-labeled serotonergic neurons (DiI/5-HT/acetylated tubulin-positive cells) in the anterior gut (Figure 2D), esophagus (Fig. 2E), typhlosole, and adjacent tissues, as well as other DiI<sup>+</sup> neurons that were serotonin negative (SupFig. 1C). These results demonstrate that neural crest cells migrating from the trunk neural tube and subsequently associated with neural processes can contribute to enteric neuron populations within the gut wall.

To determine if markers associated with neural crest-derived Schwann cell precursors were present in the lamprey trunk, we performed in situ hybridization with markers homologous to myelin genes present in the lamprey genome and characteristic of SCPs. Results reveal expression of transcripts for *Pmp22* (encoding peripheral myelin

protein 22) and *EMP2* along peripheral nerves (Fig. 3A, B). In addition, other genes associated with the Schwann cell lineage were noted in the vicinity of the ventral roots (SupFig. 3).

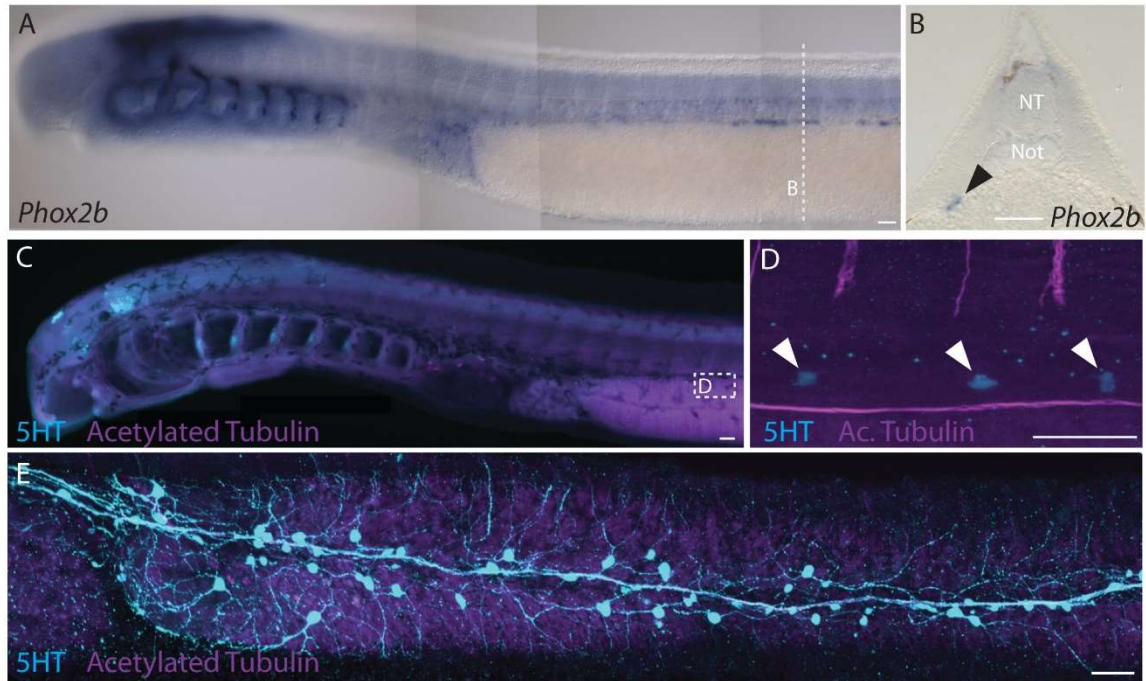
Finally, as an additional means of demonstrating that enteric neurons originated from within the trunk neural tube, we performed neural tube ablation at E6.5, a method classically used to demonstrate the neural crest origin of enteric neurons<sup>7,16</sup>. Whereas control embryos at E35 had numerous serotonergic neurons within the gut (Fig. 3C), the numbers were greatly diminished after trunk neural tube ablation (Fig. 3D). Given that the lamprey spinal cord has remarkable regenerative capacity<sup>17,18</sup>, the profound diminution in numbers of ENS neurons at the level of ablation further supports an important contribution of trunk neural tube derived cells to enteric neurons of the lamprey gut.

Recent evidence suggests that many neural crest derivatives in post-natal mammals, including skin and peripheral ganglia, arise from neural crest-derived Schwann cell precursors that are closely associated with extrinsic innervation to these structures. For example, SCPs along nerve processes have been shown to differentiate into pigment cells of the skin and parasympathetic ganglia<sup>1,2,19</sup>, including enteric neurons<sup>3</sup>. These studies definitively prove the existence of neural crest-derived cells that contribute to the peripheral nervous system and other derivatives at post-embryonic stages. Our results suggest that these trunk neural crest-derived cell types may represent an ancient and evolutionary conserved source of cells that contribute to the enteric nervous system. Moreover, our data suggest that agnathans might lack a classical “vagal” neural crest region, leading us to speculate that the neural crest coopted the ability to form enteric neurons in stem gnathostomes (Fig. 3E). Thus, the contribution of SCPs to the enteric nervous system might

represent a primitive (pleisomorphic) state retained from early vertebrates, and perhaps common to all living vertebrates. With the emergence of jawed vertebrates, new traits, including jaws, sympathetic and vagal neural crest-derived enteric ganglia appear to have come under the umbrella of embryonic neural crest derivatives.

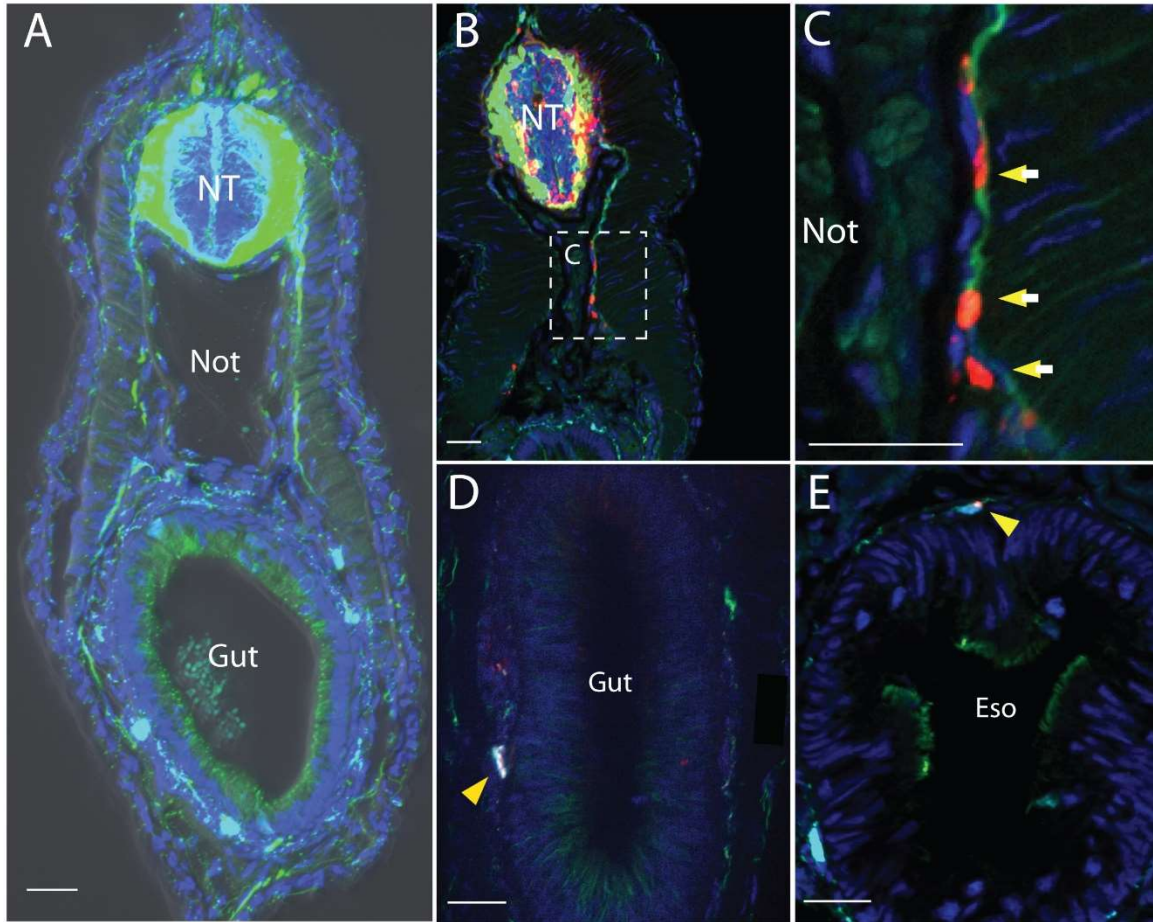
### **Methods Summary**

Adult lamprey were supplied by the US Fish and Wildlife Service and Dept of the Interior, and cultured according to previous protocols<sup>20</sup>, and in compliance with IACUC protocol #1436. DiI from Invitrogen was resuspended as described<sup>8</sup>, and surgical ablations were performed using forceps and tungsten needles. In situ hybridizations were performed as described and antibody staining was performed using previously described protocols<sup>21,22</sup>. Anti-acetylated tubulin (Sigma; Mouse IgG2b) was used at a 1:100 dilution, anti-5-HT (Immunostar; Rabbit IgG) was used at 1:500. Microscopy was performed on a Zeiss Imager equipped with an Apotome. Embryos were processed for cryosectioning according to standard protocols, and were processed with a *Microm* HM550 *cryostat*.

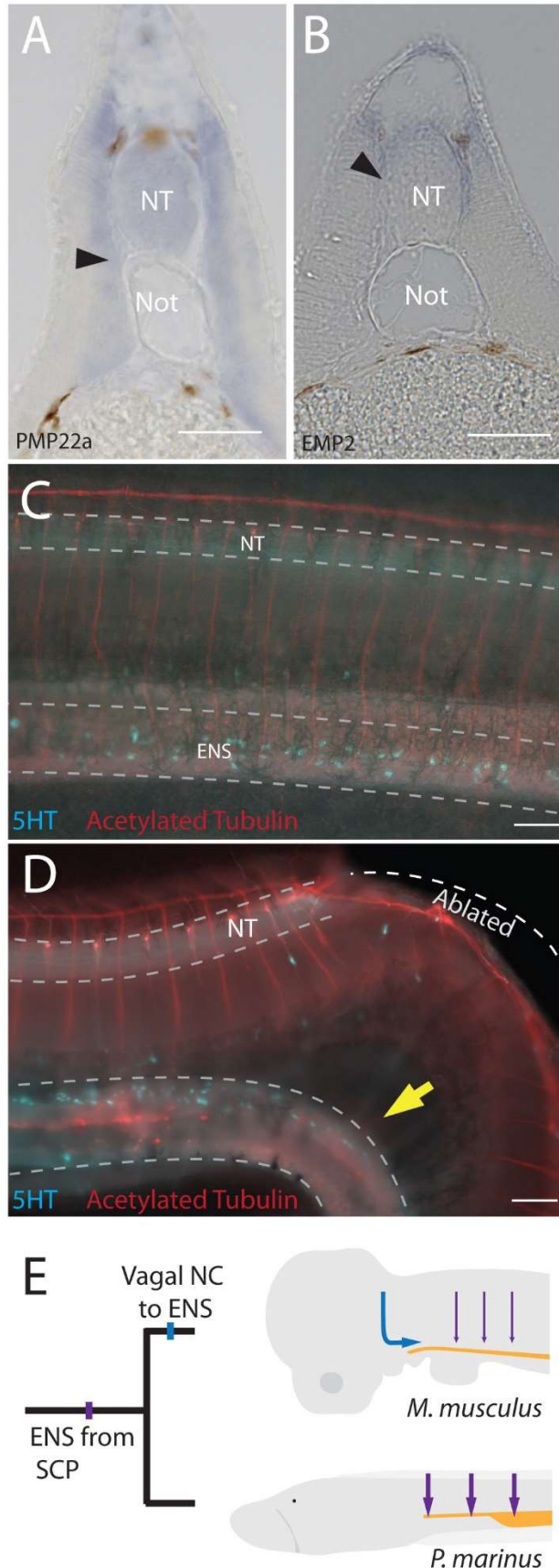


**Figure 1. Early formation of enteric neurons in the lamprey *P. marinus*.** A: *Phox2b* expression in dorsolateral cells of a T28 embryo. B: Transverse cross-section of a T28 embryo shows *Phox2b* expression in the depression of the typhlosole (black arrowhead). C: 5-HT and acetylated tubulin immunoreactivity in a slightly older T28.5 embryo. D: 5-HT is detectable in neurons (white arrowheads) adjacent to the vagus nerve. E: Serotonergic neurons form small ganglia within the enteric plexus of an E30 embryo. NT: neural tube; Not: notochord. All scale bars are 20  $\mu$ m.





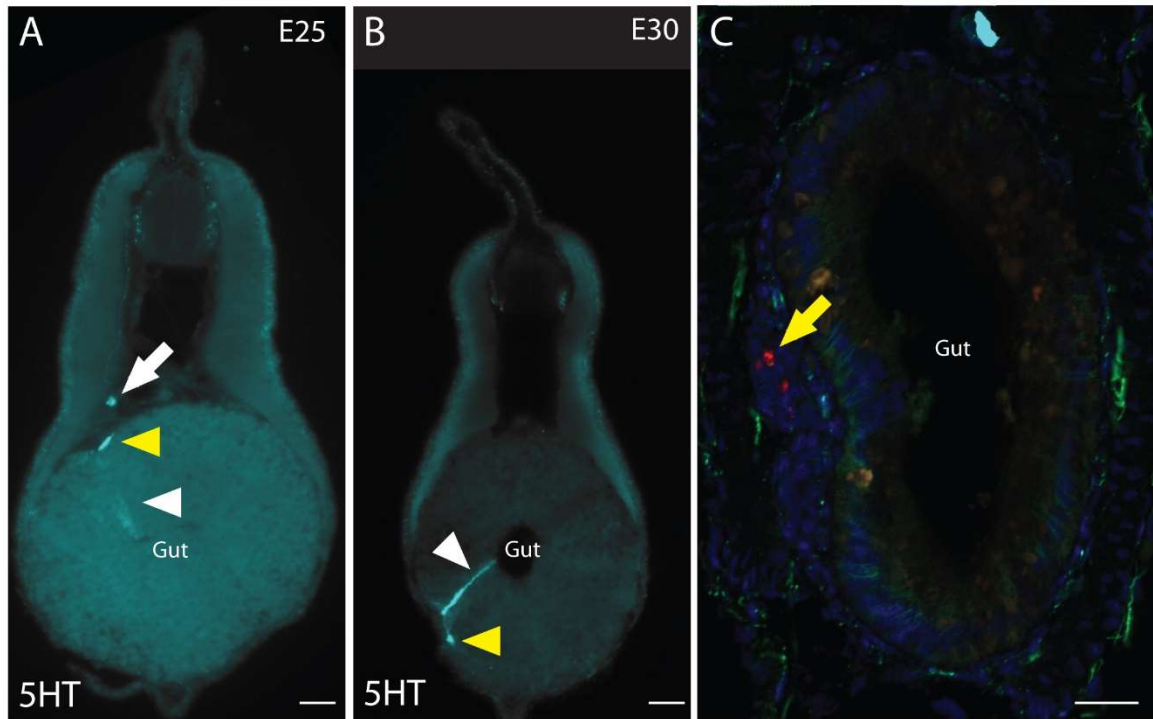
**Figure 2. DiI Labeled cells in the neural tube contribute to enteric ganglia.** Immunohistochemistry of E30 Lamprey transverse cryosections. A) Uninjected control highlighting neuronal projections and serotonergic ganglia around the gut. Red=Acetylated tubulin; Blue=DAPI; Cyan=5-HT. B-D) DiI injected “tubefill” embryos. Red=DiI; Green=Acetylated Tubulin; Cyan=5-HT; Blue=DAPI. B & C) DiI within the neural tube, dorsal root ganglia, and along spinal projections. D) A DiI-labeled enteric neuron (acetylated tubulin<sup>+</sup>, 5-HT<sup>+</sup>) within the typhlosole of the gut wall. E) A DiI-labeled neuron (acetylated tubulin<sup>+</sup>, 5-HT<sup>+</sup>) in the esophagus. Eso: esophagus; NT: neural tube; Not: notochord. All scale bars are 20  $\mu$ m.



**Figure 3. Schwann cell precursors contribute to the ENS.** A & B) *In situ* hybridization of Schwann cell markers *Pmp22a* and *EMP2* show domains of expression along spinal nerves in transverse cryosection. C & D) Immunohistochemistry on wholemount E30 lamprey (Red:HuC/D and acetylated tubulin; Cyan:5-HT) C) Control unablated embryo. D) Ablation of the neural tube at E6.5 results in a decrease of serotonergic (5-HT<sup>+</sup>) cells in the adjacent gut. E) Model of neural crest enteric contributions to the ENS in lamprey and mouse. All scale bars are 50  $\mu$ m.

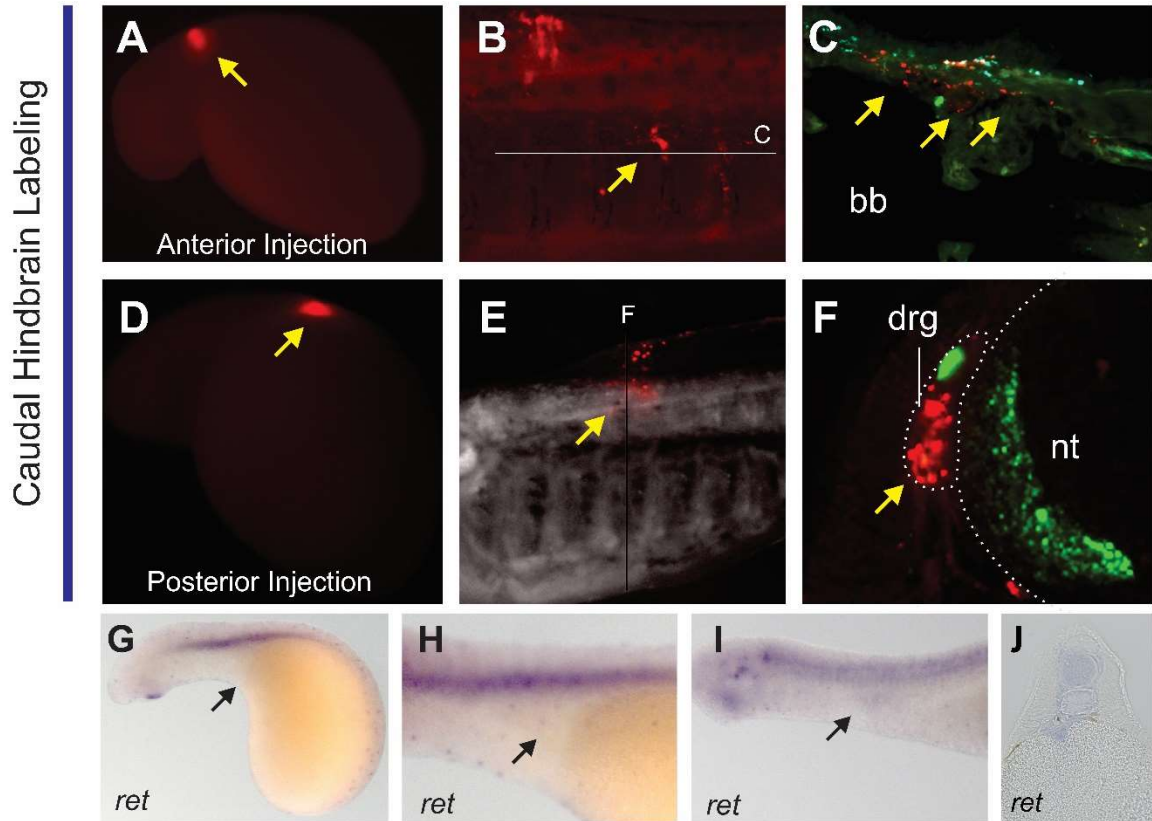
Table 1. Number of Dil-labeled neurons (AcTub+) in 25 representative E30-35 embryos in which Dil had been injected into the neural tube at T22.5.

Location	Dil+, 5HT+	Dil+, 5HT-	Total Dil-labeled Neurons
Esophagus	22	2	24
Intestine (Basal Typhlosole)	26	0	26
Intestine (Typhlosole mesenchyme)	4	5	9
Total	52	7	59

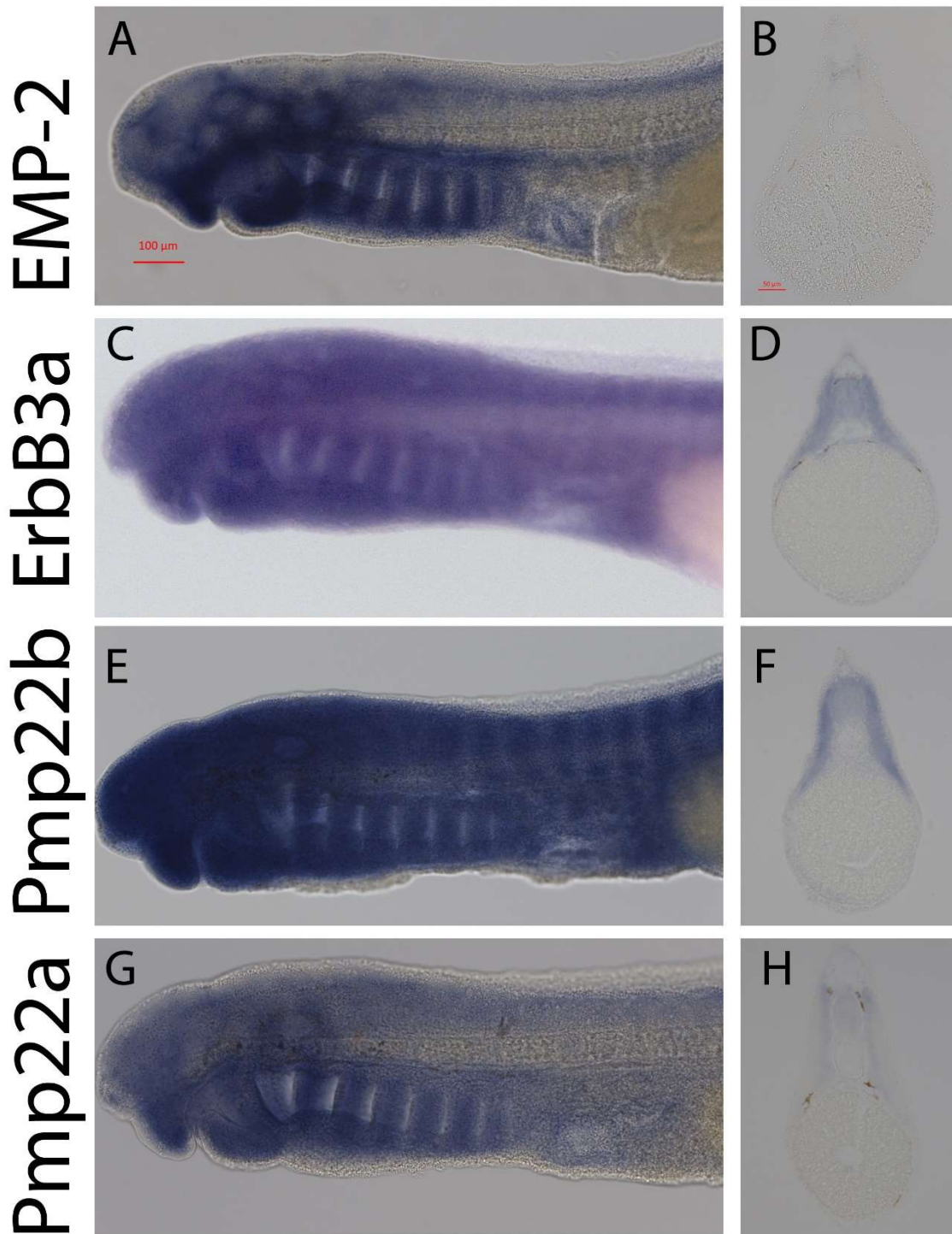


**Supplemental Figure 1: 5-HT immunoreactive cells in the gut, and DiI labelling.** A-B: 5-HT Immunoreactivity for 5-HT in E25 (A) and E30 (B) embryos. Serotonergic neurons (yellow arrowheads) are positioned within the typhlosole, adjacent to the endodermal mucosa. A cell present in the columnar epithelia (white arrowhead) might represent an enterochromaffin-like cell. Cells positioned dorsal to the typhlosole, near the nephric folds, are probably unrelated nephridial autonomic neurons (white arrow). C: Di-I labeling results in labeled cells (yellow arrowhead) migrating to the gut and typhlosole. All scale bars are 20  $\mu\text{m}$ .





**Supplemental Figure 2: DiI labeling of the caudal hindbrain population shows contributions to the branchial arches but not outflow tract or gut.** A-C and D-F: Sample time lapse imaging of two separate DiI labeled embryos around the cervical level. A & D) Initial injection at E6-E6.5. B) Final DiI localization of embryo in A 10 days post injection (E16). C) Frontal cryosection through the branchial basket shows DiI along the branchial arches. Red: DiI, Green: Neurofilament-M, Cyan: Collagen type II. E) Final DiI localization of embryo in A 14 days post injection (E20) F) Transverse section through the lamprey branchial basket shows DiI within the drg. G-I) E8-E10 Ret *in situ* hybridization showing the absence of ret in the presumptive vagal/cardiac region J) Transverse section through a T27 Ret embryo. bb:branchial basket; drg:dorsal root ganglia; nt: neural tube. Red: DiI; Green: Neurofilament-M.



**Supplemental Figure 3: Examining schwann cell markers in lamprey.** *In situ* hybridization examining schwann cell genes in lamprey whole mount and cross section at T26. A & B) EMP-2. C & D) ErbB3a, E & F) Pmp22b, and G & H) Pmp22a. Wholemound scale bars are 100 µm and cross section scale bars are 50 µm.

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*Chapter 4***NOVEL ROLE OF SOX FACTORS IN NEURAL CREST  
FORMATION**

*Chapter 4 - 1*

**EXPRESSION OF SOX FAMILY GENES IN EARLY LAMPREY  
DEVELOPMENT**

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**Abstract**

Members of the Sox (Sry-related high mobility group box) family of transcription factors play a variety of roles during development of both vertebrates and invertebrates. A marked expansion in gene number occurred during emergence of vertebrates, apparently via gene duplication events that are thought to have facilitated new functions. By screening a macroarrayed library as well as the lamprey genome, we have isolated genes of the Sox B, D, E, and F subfamilies in the basal jawless vertebrate, the lamprey. The expression patterns of all identified Sox genes were examined from gastrulation through early organogenesis (embryonic day 4-14), with particular emphasis on the neural crest, a vertebrate innovation. Coupled with phylogenetic analysis of these Sox genes, the results provide insight into gene duplication and divergence in paralog deployment occurring during early vertebrate evolution.

## Introduction

Many transcription factors of the Sox gene family are critical for a number of developmental processes, most notably sex determination, neural crest development, and neurogenesis (Laudet, Stehelin et al. 1993, Hong and Saint-Jeannet 2005, Betancur, Bronner-Fraser et al. 2010)). This family is composed of more than 30 genes that have been classified into eight paralogy groups [SoxA-SoxH; (Schepers et al., 2002)]. Sox genes are characterized by the presence of a single High Mobility Group box (HMG box), a 79 amino acid DNA binding domain that has affinity to the WWCAAW motif (Laudet et al., 1993). These factors are generally expressed in a dynamic, tissue specific manner, and often interact with other transcription factors (Prior and Walter 1996)).

Members of the Sox gene family are found across the animal kingdom, and it has been suggested that the major Sox groups (i.e. B, C, D, and E) were already present in the common bilaterian ancestor, whereas group A genes are specific to mammals (Jager, Queinnec et al. 2006). Expansion in the number of vertebrate Sox genes is thought to be due to major gene duplication events, initially occurring during early stages of metazoan evolution and later during the transition between non-vertebrate chordates and vertebrates (Prior and Walter 1996); Dehal et al., 2005). Gnathostomes (jawed vertebrates) have undergone two rounds of whole genome duplications (Escriva et al., 2002), whereas estimates for the number of rounds of duplication in agnathans like lamprey range from one to two. It is not yet clear whether supernumerary copies of lamprey genes arose via whole genome-wide duplication, or via independent duplication events (Tomsa et al., 1999; McCauley et al., 2006; Neidert et al., 2001; Zhong et al., 2011).

Classification of Sox genes was first done by Wright et al., 1993, using partial sequences from mouse SOX genes. This study defined the six paralogous groups (A-F) which are the basis of the current classification (Wright et al., 1993). Four more groups were subsequently added to include recently identified paralogs (Bowles, Schepers et al. 2000). Nevertheless, members of the same groups do not always have similar roles or expression patterns, indicating that recent paralogs can adopt new functions with relative ease (Bowles et al., 2000).

In the neural crest, a vertebrate innovation that contributes to the peripheral nervous system and craniofacial skeleton, the function of Sox genes has been studied at many stages. For example, gnathostome SoxE family members (Sox8, Sox9, and Sox10) are expressed in premigratory neural crest progenitors and migrating neural crest cells, as well as at later stages in numerous neural crest derivatives (Sauka-Spengler et al., 2008), similar to their lamprey paralogs (McCaulsey et al., 2006) SoxD family members (Sox5 and Sox6) are found in cranial ganglia (Morales et al., 2007) and cartilage elements of neural crest origin. Additionally, gnathostome SoxB family members have been shown to play an essential role in differentiation of late neural crest derivatives (Wakamatsu et al., 2004) (Wakamatsu, Endo et al. 2004).

Given the important function of Sox genes in gnathostomes, we sought to identify and characterize novel lamprey genes of the SoxD and SoxF subfamilies, as well as additional members of the SoxB and SoxE groups. As one of the basal-most extant vertebrates, analysis of the deployment of paralogous genes in lamprey offers the opportunity to examine events in early emergence of vertebrate specific features. Comparative amino acid analysis between lamprey and other vertebrate Sox genes provides insight into the

evolutionary history of early vertebrates, as well as duplication events occurring early in the vertebrate lineage.

## **Materials and Methods**

### ***Heterospecific screening of an arrayed lamprey embryonic cDNA library***

A high quality directional full-length arrayed cDNA library (Sauka-Spengler et al., 2007) from embryonic day 2-12 lamprey embryos (an average efficiency of  $\sim 0.9 \times 10^8$  transformants/ $\mu\text{g}$  of cDNA) was used for low-stringency screening. Nine individual nitrocellulose filters were screened using Sox heterospecific probes, yielding 7 different Sox genes (SoxB, E, and F family members), whose identity was confirmed by sequencing on both strands, BLAST searching, and phylogenetic analysis (see below).

### ***RNA-ligated mediated 5' Rapid Amplification of cDNA ends (RLM-5' RACE)***

A SoxD homologue was identified by bioinformatic survey of the lamprey genomic sequences and cloned using RACE. RACE was also used to obtain full-length sequences of the Sox genes, where cDNA clones were incomplete. Total RNA was extracted from 6, 8, 10, and 14 day old embryos from Ambion:RNAqueous kit. RLM-5' RACE was conducted on the total mRNA in accordance with Invitrogen: GeneRacer Kit. Total RNA was dephosphorylated through Calf Intestinal Phosphatase (CIP) treatment, decapped via Tobacco Acid Pyrophosphatase (TAP), ligated with the GeneRacer RNA oligo, and finally

reverse transcribed using random hexamer priming to form the cDNA template. Gene specific primers were:

GeneRacer 5' Primer CGACTGGAGCACGAGGACACTGA 3'

SoxB1-B: 5' CGACTGGAGCACGAGGACACTGA 3'

SoxD: 5' CGCCTCTCGTCCTTTGCCCAGAC3'

### ***Touch Down PCR***

The touchdown PCR procedure was based off of the Invitrogen GeneRacer Kit. Samples were prepared in 5% DMSO, using TAQ polymerase with Roche Expand Long Template PCR Buffer 1(10x concentrated, 17.5 mM MgCl<sub>2</sub>). A hot start at 94°C was conducted followed by 5 cycles of 94°C for 30 seconds and 72°C for 3 minutes. Another cycle of 94°C for 30 seconds and 70°C for 3 minutes was done proceeded by 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes for 36 cycles, and a final extension at 72°C for 10 minutes.

### ***Cloning of the PCR product***

Extraction of the PCR product was conducted corresponding to Qiagen:QIAquick Gel Extraction Kit and cloned with Invitrogen: TOPO TA Cloning. The clones were selected against the metabolism of X-gal and the production of  $\beta$ -galactosidase purified following the QIAprep spin miniprep kit and sent for sequencing. (Davis Sequencing, Davis, CA)



***Embryo collection and maintenance.***

Mature or maturing *Petromyzon marinus* adults were obtained from Hammond Bay Biological Station, Millersburg, MI, USA. Mature animals were maintained in our lamprey facility in 12°C chilled re-circulating water and used for spawning. Juveniles were kept at lower temperatures before the onset of the maturation and then progressively induced to maturation by gradually augmenting the water temperature and expanding the daylight cycle. For *in vitro* fertilization, eggs are stripped manually from a single gravid female into a 500ml crystallizing dish containing 100-200ml of spring water and milt from a spermated male is then expressed directly onto the eggs. After 15 minutes, the fertilized eggs are washed through several changes of distilled 18°C water and placed in a 4-liter container in spring water in the 18°C incubator. After the first division the embryos are transferred to 0.1X MMR (Marc's Modified Ringers) for long-term culture. The medium from each culture is replaced with fresh media every day to avoid fungal infection. Embryos were fixed in MEMFA (4% formaldehyde, 0.1M MOPS (pH 7.4), 1 mM MgSO<sub>4</sub>, 2 mM EGTA), dehydrated gradually, and stored in 100% methanol at -20°C (Sauka-Spengler et al., 2007).

***In situ hybridization and histology.***

Whole-mount in situ hybridization of lamprey embryos was performed using digoxigenin- or RNA probes according to Xu and Wilkinson (Xu et al., 1998), with the following modifications: prior to Proteinase K step, embryos equilibrated in the bleaching solution (0.5X SSC, 5% formamide, 10% H<sub>2</sub>O<sub>2</sub>), were exposed to direct light using a light box for 10-15 minutes. The concentration and the length of Proteinase K treatment

(~20µg/ml, 10 minutes) was the same for embryos of all stages. Hybridization and subsequent washes were carried out at 70°C in hybridization solution containing 50% formamide; 1.3X SSC; 5mM EDTA pH8.0; 200 µg/ml yeast tRNA; 100µg/ml heparin; 0.2% Tween-20 and 0.5% Chaps. The hybridization signal was detected using BM Purple substrate (Roche, Indianapolis, IN) for early stage embryos (E3-E10) or NBT/BCIP (Roche, Indianapolis, IN) for later stages. After photographing, embryos were post-fixed in 4% Paraformaldehyde/PBS, rinsed in PBS, cryo-protected in two subsequent steps: 15% sucrose/PBS and 7.5% gelatin/15% sucrose/PBS, equilibrated and mounted in 20% gelatin/PBS and frozen in liquid nitrogen. 10µm cryosections were collected on Super Frost Plus slides (Fischer Scientific, Pittsburgh, PA).

### ***Phylogenetic Analysis***

The amino acid alignments and Neighbor Joining (NJ) tree were constructed using ClustalX. The Maximum Parsimony likelihood tree was built using Mega. The trees were visualized using Tree View v. 0.5.0. Protein sequences from the HMG boxes of Sox family genes were used to build the alignments. Sequences from other species were retrieved from GenBank, and carry the following nomenclature abbreviations: *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Mm*, *Mus musculus*; *Pm*, *Petromyzon marinus*; *Xl*, *Xenopus laevis*.

## Results and Discussion

### *Phylogenetic Analysis*

The screening of a full-length cDNA library (Sauka-Spengler, Meulemans et al. 2007) coupled with BLAST searches of the lamprey genome, allowed for identification of four new lamprey Sox orthologs, from Sox families B, D, E, and F. We also included in our analysis the three SoxE orthologs previously described in McCauley et al., 2006 (McCauley and Bronner-Fraser 2006).

Three SoxB genes were identified in this study: SoxB1b, SoxB2 and a putative SoxB1a gene. The lamprey SoxB2 groups with the Sox 21/14 in our phylogenetic analysis, while SoxB1-A clusters with the Sox1/2/3 paralogy group. The presence of orthologs of SoxB1 and SoxB2 in the lamprey is expected since the duplication that gave rise to these genes is thought to be ancient, having occurred before the deuterostome/protostome split (Mckimmie et al., 2005; (Zhong, Wang et al. 2011)). The putative SoxB1a gene we have cloned seems to be an ortholog of the Sox2 gene due to the similarity of the 5' fragment of the transcript. This gene lacks a HMG box, which precludes a more detailed analysis of its phylogenetic position. However, the expression data obtained for this gene (see below) suggests that it is likely related to the other SoxB genes analyzed.

We identified only a single SoxD family in lamprey that is apparently quite divergent from the other vertebrate SoxDs and more similar to Sox6 group. However, this may be an artifact due to the large number of amino acid substitutions observed in this ortholog (Figure 1, S1). The lamprey SoxF, on the other hand, clusters in the base of the Sox7/18/17 branch. Thus, our data suggest that there is one lamprey ortholog for half of

the major Sox families (D, F, B2), suggesting that the duplication events that led to the expansion of paralogs in such families took place after the Cyclostome/Gnathostome split.

### ***Expression pattern of SoxB family members***

The SoxB family is known to play a major role in neural induction and differentiation. In zebrafish, SoxBs are expressed in an early stage starting at the anterior neural plate and throughout the CNS (Rauch et al., 2003; Thisse et al, 2005). Whole mount *in situ* hybridization of the SoxB family revealed that SoxB1a, SoxB1b, and SoxB2 are all expressed in the neural plate at embryonic day (E) 4 (*Figure 2: A, B & C*). Similarly at E5, SoxB1a and SoxB1b have expression patterns in the neural tube with the exception of its anterior-dorsal aspect (*Figure 2: A & B*), whereas SoxB2 is expressed continuously throughout the dorsal neural tube (*Figure 2: C*). At E6.5, by which time neural crest cells are within the dorsal aspect of the neural tube preparing to emigrate, SoxB1a and SoxB2 are expressed on the dorsal aspect of the neural tube (*Figure 2: A & C*) while SoxB1b is absent from the anterior-dorsal region (*Figure 2: B*). At E8, SoxB genes are expressed in the neural tube as well as in the forming branchial arches. Analysis of sectioned embryos reveals that SoxB2 is expressed mainly in the neural tube, the cranial ganglia, trigeminal ganglia, and in cardiac tissue.

Finally, between E10-E14, the SoxBs are expressed in the neural tube and in the brachial arches. At E14, SoxB1a and SoxB1b are expressed in the branchial arches. Similarly, SoxB2 is expressed in both neural tissue and mesoderm-derived portions of the branchial arches. SoxB2 is also expressed in cardiac and other non-neural crest derived tissue. These data suggest that the SoxB genes are expressed in similar domains but in a

distinct temporal sequence, first with SoxB2, followed by SoxB1a and then SoxB1b. Despite their staggered temporal expression, all SoxBs are present in migrating neural crest cells as well as neural crest derivatives such as cranial ganglia and branchial arches. SoxB2 is present in both premigratory and migrating neural crest cells.

In *Xenopus*, SoxBs play a role in neural plate formation and also have later roles throughout CNS formation (Cunningham et al., 2008; Kishi et al., 2000, Rodgers et al., 2008). In chick, SoxBs are also expressed on the neural plate and along the neural tube. In mice, SoxBs are all expressed in the primitive streak ectoderm. Sox1 is expressed early on in the neural folds. Sox 2 and 3 are found in the forming neural plate onward (Wood et al., 1999).

### ***Expression pattern of SoxD family members***

SoxDs are expressed widely in vertebrate neural tissues, forebrain, in fast muscles, somites, myotome, and cardiac precursors (Wang et al., 2011; Von Hofsten et al., 2008; Kudoh et al., 2001). SoxDs are necessary for formation of the notochord and chondrogenesis. They are also found in glial cells and other early NC lineages (Smits & Lefebvre, 2003; Lefebvre et al., 1998; Perez-Alcala et al., 2004). In lamprey, SoxD is expressed at high levels on the neural plate border and along the neural folds at E4 (*Figure 3: A*). At later stages, SoxD is not expressed in the premigratory crest but is expressed along the neural tube (*Figure 3: B*). At E8, it is observed in the optic vesicle (*Figure 3: D & D'*). Beginning at E10, SoxD is expressed in the endoderm- and mesoderm-derived portions of the branchial arches (*Figure 3: E, E', & E''*). From E12-E16, the mesenchymal portions of the branchial arches condense to form the branchial cartilage (*Figure 3: F-H &*

*F''-H'*). Interestingly, at E14, SoxD is heavily expressed in the heart but not at prior or subsequent stages.

### ***Expression pattern of SoxE family members***

The SoxE family has three subgroups denoted SoxE1, E2, and E3 (McCauley and Bronner-Fraser, 2006). At E4, SoxE1 and SoxE2 both display low levels of expression in the neural plate (*Figure 4: A & H*) whereas SoxE3 is not yet expressed (*Figure 4: O*). By E5, all SoxEs exhibit expression in the neural tube as well as distinct domains in the embryo. At E5-E7, SoxE1 is observed in two regions of the anterior-dorsal aspect of the neural tube (*Figure 4: B-D*). At later stages, it is expressed in the neural tube, cranial ganglia (*Figure 4: D & D'*), dorsal root ganglia (*Figure 4: F & F'*), and in mesoderm and neural crest derived portions of the branchial arches cartilage (*Figure 4: G & G'*). SoxE2 is expressed similarly but with higher intensity (*Figure 4: I-K*). At older stages, SoxE2 is expressed in the neural tube, cranial ganglia (*Figure 4: K-K'*), optic (*Figure 4: L & L'*), otic vesicles (*Figure 4: M & M'*), and branchial arches (*Figure 4: N-N''''*). SoxE3 exhibits a strong signal starting at E5 in the optic vesicle (*Figure 4: P*). It is strongly expressed during neural crest migration. From E6-E14, SoxE3 is very prominently expressed in the otic vesicles (*Figure 4: Q-S, & S'*), cranial ganglia (*Figure 4: S*), neural tube, and branchial arches (*Figure 4: S-U & S''-U''*).

### ***Expression pattern of SoxF family member***

In vertebrates, SoxFs are involved with vascular development and cardiogenesis, and expressed in structures such as the aortic arch, circulatory system, and pharyngeal arches in

addition to parts of the CNS (Zhang et al., 2005; Kyuno et al., 2008). We isolated a single SoxF gene. At gastrula stages, SoxF is expressed in involuting cells in the dorsal lip of the blastopore (*Figure 5: A & B*). At the neurula stage (E4.5), SoxF is found in the neural plate (*Figure 5:C*). Expression in precursor cells to the thyroid gland begins at E7, whereas by E8, SoxF is observed in tissues surrounding the notochord (*Figure 5: D, E and L*). Low levels of SoxF are observed in cardiac precursors at E10 (*Figure 5: I*). At E12, SoxF is more strongly expressed in the anterior branchial arches, similar to that noted for SoxD (*Figure 5: J*).

## Conclusion

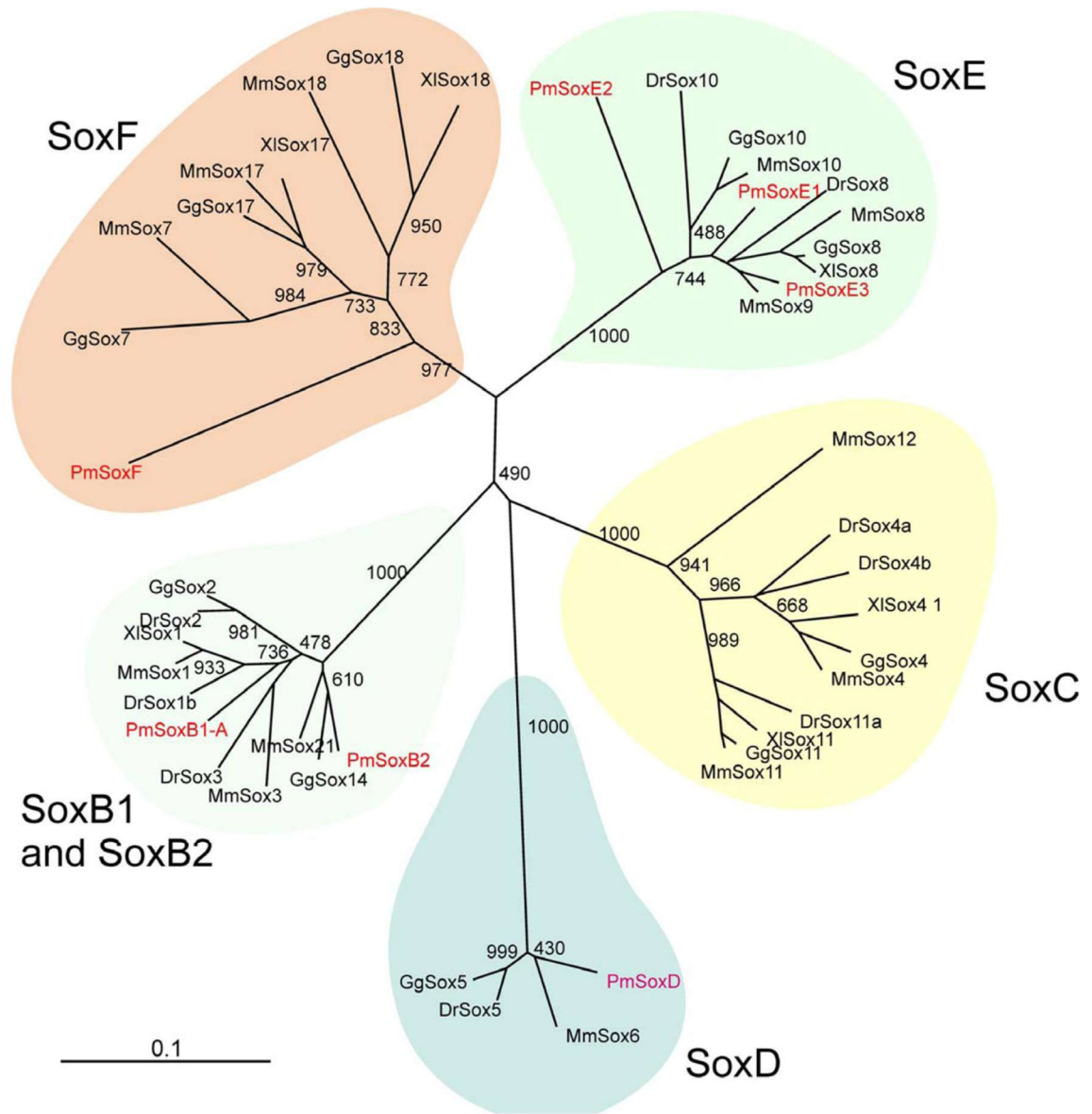
Taken collectively, our phylogenetic and expression data provide interesting insights into the evolution of Sox genes in vertebrates. Surprisingly, we were only able to find one ortholog for half of the major Sox families (D, F, B2). This suggests that either these families expanded after the emergence of jawed vertebrates, or that there was extensive loss of these Sox genes in lamprey. For the remaining families, the Sox genes appear to have undergone independent duplications though there may be some bias toward particular paralogs. For example, SoxE1 is most similar to Sox9 and SoxE2 to Sox8. Based on the positions of various genes, we speculate that the SoxB2 gene (Sox14) and SoxD genes either diverged earlier than their gnathostome orthologs or that paralogs were lost, as seen by Sox14 and Sox 5a or 6. SoxF is very similar to all variants of SoxF in jawed vertebrates, suggesting it may be an ancestral gene that was independently duplicated by jawed vertebrates.

Our expression data further support the idea that lamprey genes underwent independent gene duplications, separate from the two rounds of gene duplication observed in other vertebrates. For example, in those Sox families in which we identified multiple members, we find that the lamprey genes often have overlapping expression patterns, the sum of which reflects the overall expression pattern of that Sox family in other vertebrates. Generally, each family has a high degree of overlapping expression. However, no unique ortholog was found with a pattern that is similar only to a single gnathostome Sox gene, except when we found only a single member of that family in lamprey. Sequencing of additional cyclostome genomes will provide clarity as to when such independent duplications may have occurred.

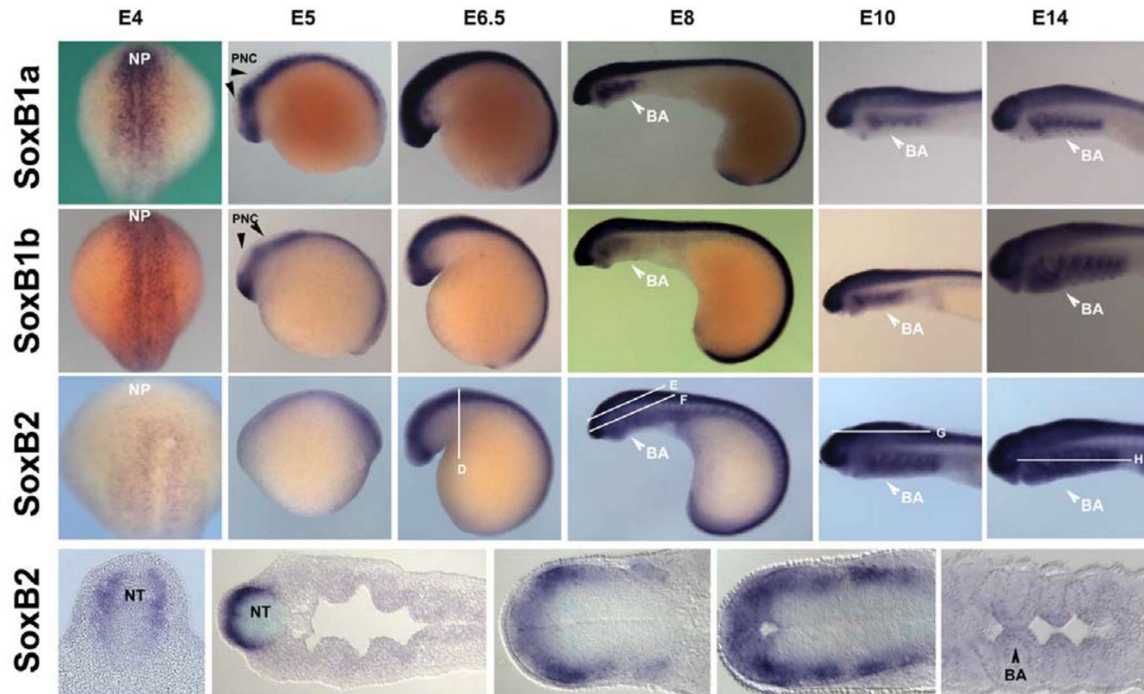
### **Acknowledgement**

We thank Natalya Nikitina for her contribution of previously sequenced genes. This work was supported by NIH grant DE017911 to MEB.

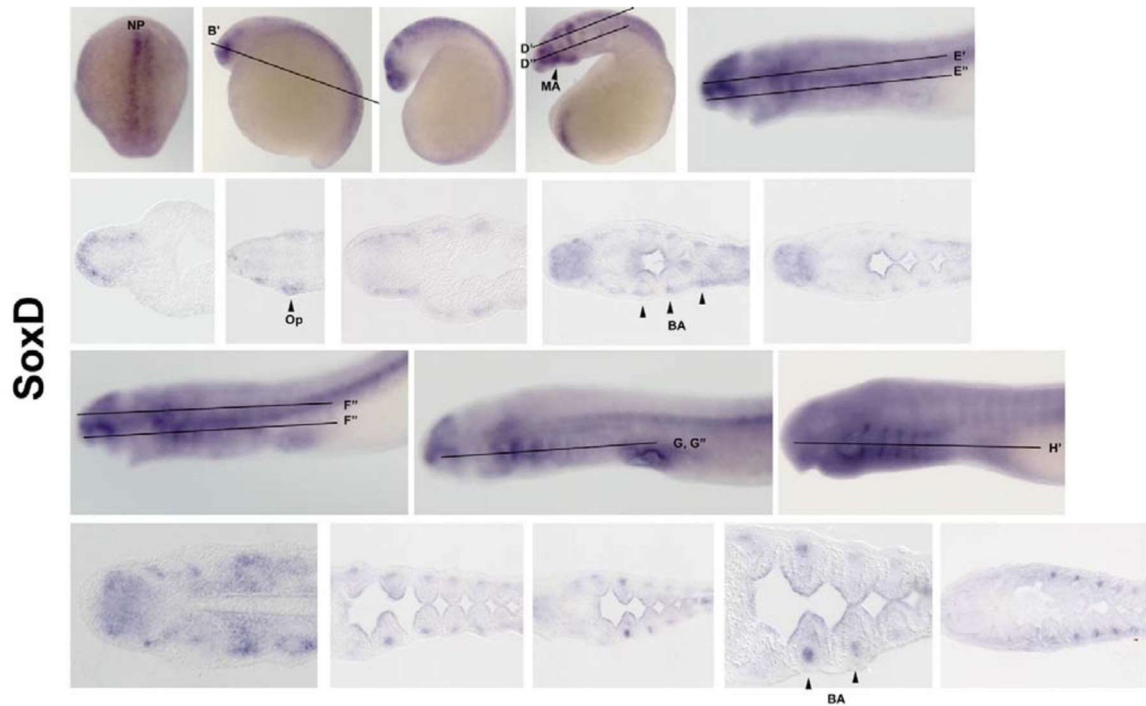




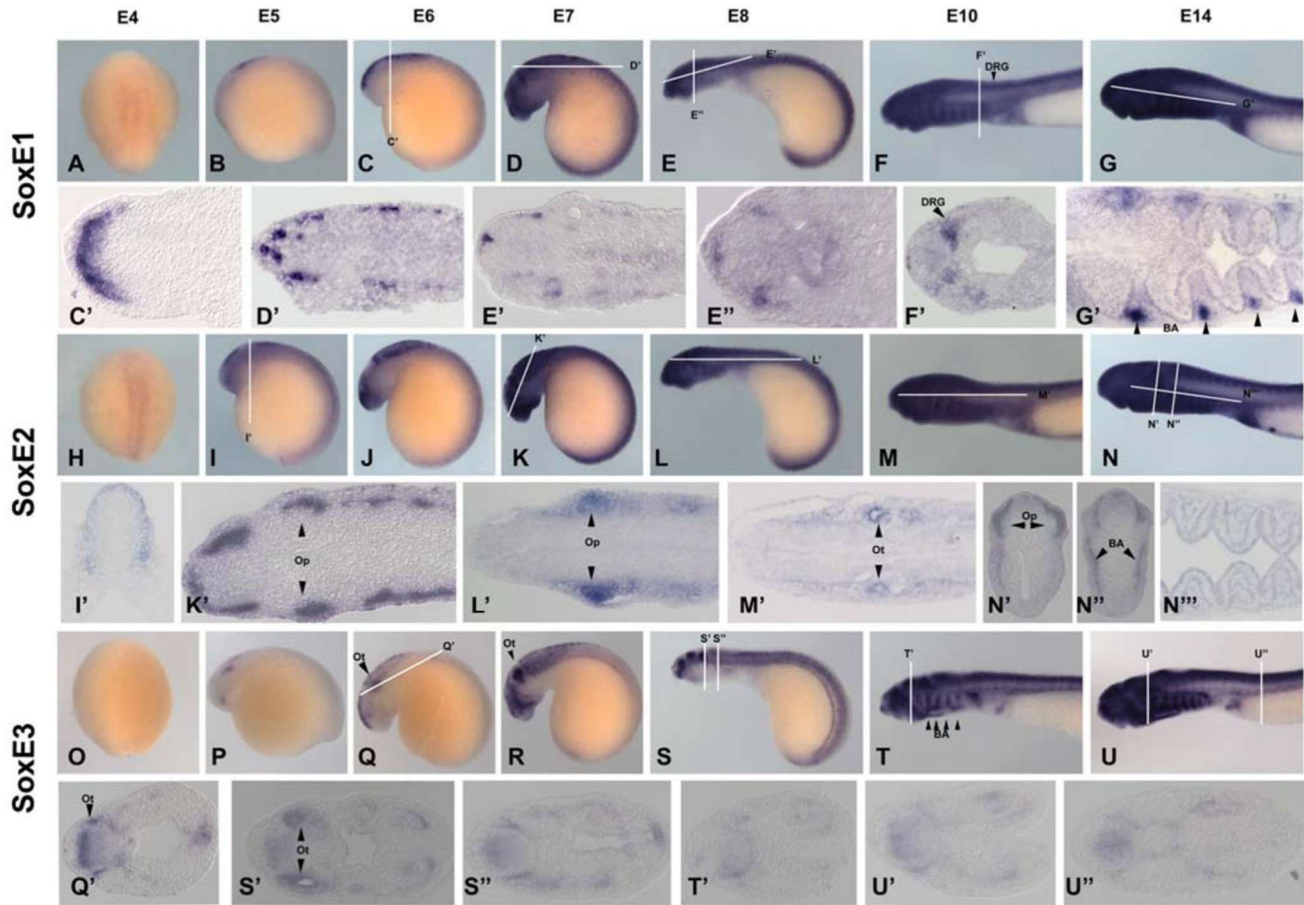
**Figure 1: Phylogenetic Analysis of lamprey Sox genes:** Unrooted phylogenetic tree of Sox genes obtained from a neighbor joining analysis performed on ClustalX. The HMG box protein sequences were used in the construction of the tree. Boxes highlight the families of Sox genes. Abbreviations are *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Mm*, *Mus musculus*; *Pm*, *Petromyzon marinus*; *Xl*, *Xenopus laevis*.



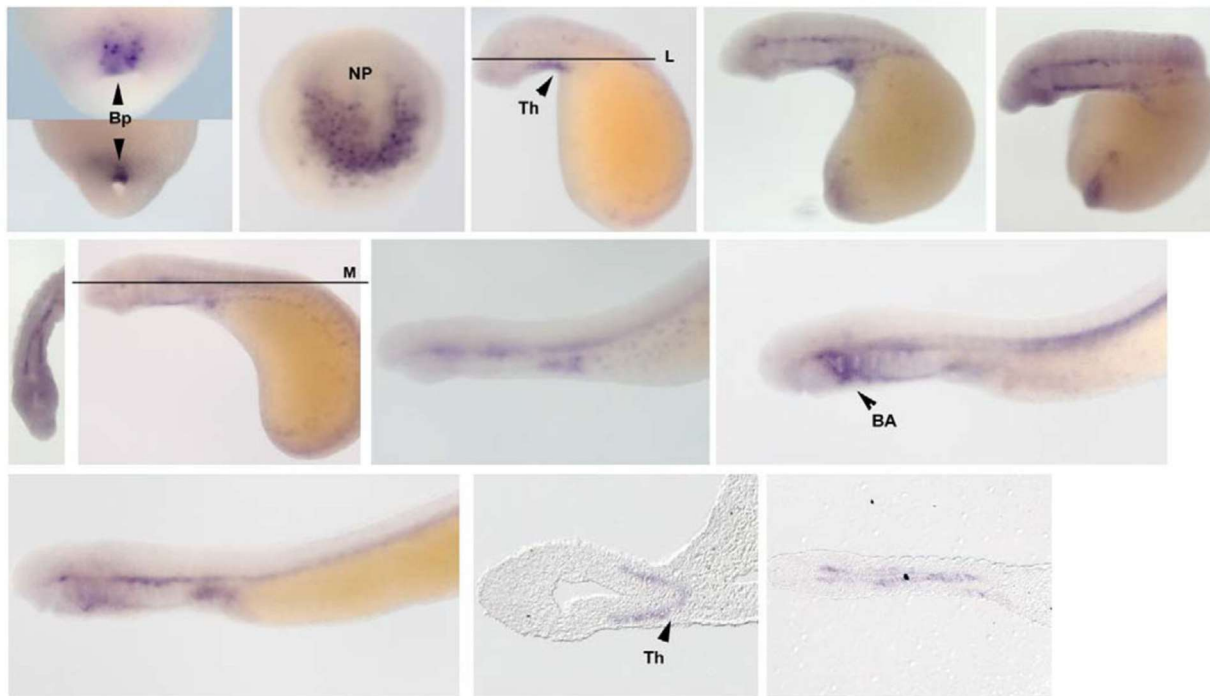
**Figure 2: SoxB Expression:** The SoxB genes are expressed within the neural plate, neural tube and branchial arches. SoxB1a, 4-14 days (A): SoxB1a is not expressed in pre-migratory neural crest at E5, with expression initiating at E6 at the onset of neural crest emigration. SoxB1b: 4-14 day (B) SoxB1b is not expressed at E5 or E6 in pre-migratory neural crest. SoxB2; 4-14 day, (C) and sections (D-H) from 6.5 day, 8 day, 10 day, and 14 day, is expressed throughout the neural tube at E5 and E6. Sections reveal SoxB2's presence at later stages in the neural tube, cranial ganglia, and ectoderm derived portions of the branchial arches. NP: neural plate, PNC: Pre-migratory neural crest, NT: neural tube, BA: branchial arch.



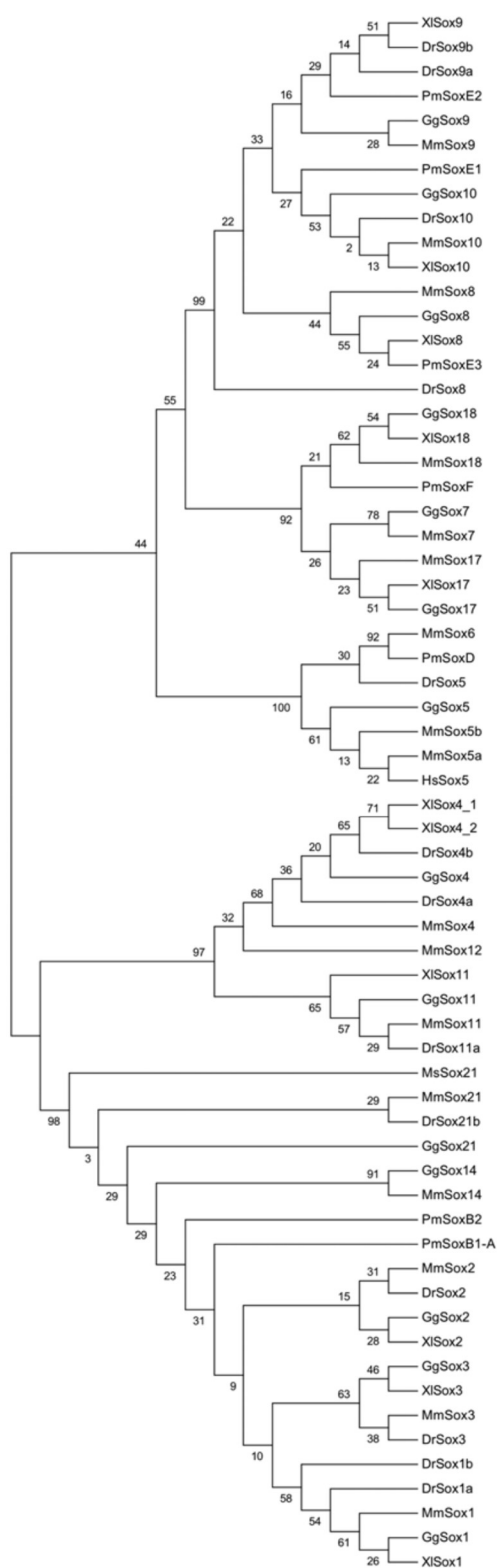
**Figure 3: SoxD Expression:** SoxD is expressed from 4 - 16 day (A-H), sections (B'-H') beginning on the boarder of the neural plate(A) and progressing to various neural crest derivatives, optic vesicle(D & D'), branchial arches (E, E' & E''), and branchial arch cartilage(F-H & F''-H'). Beginning E8 (D) SoxD is expressed in forming branchial arch progenitors, optic vesicle and mandibular arch. From E10-E16(E-H), expression in the forebrain and heart are down decreased over time while expression of SoxD is consistent with branchial arch cartilage condensation. Np: neural plate, Op: optic vesicle, BA: branchial arch.MA: mandibular arch.



**Figure 4: SoxE Expression:** The SoxE family show low level of early expression with SoxE1 and E2 expression in the neural plate at E4 (A & H). SoxE1(A-G) begins showing expression in the pre-migratory neural crest (C & C') then cranial ganglia (D,D',E,E', & E''), dorsal root ganglia (F & F'), and the branchial cartilage at late stages(G & G'). SoxE2(H-N) is also expressed heavily in the premigratory neural crest (I & I') and ultimately in the optic (K,K', L, L' and N'), otic vesicles (M&M'), and branchial arches (N-N'''). SoxE3(O-U) is expressed early in the otic vesicle (Q & Q'). It is later expressed heavily in the otic vesicle (S & S'), other cranial ganglia (S-U), neural tube, branchial arches(S-U). Op: optic vesicle, Ot: otic vesicle, BA: branchial arch



**Figure 5: SoxF Expression:** SoxF is expressed early within the blastopore [(A & B) ventral view; E4.5] and the neural plate (C dorsal view). Beginning from E7 to E14, SoxF is expressed surrounding the notochord and in precursors to the thyroid, heart, and branchial arches (E-M). Beginning at E7, SoxF begins to express in the forming thyroid (L). Ventral view of E8.5 displaying the forming thyroid (G). In addition, SoxF is temporarily expressed in the first and second branchial arch at E14 and 16 (J and K). Bp: blastopore, NP: neural plate Th: thyroid precursors BA: Branchial Arch.



**Figure S1: Maximum Likelihood phylogenetic tree of lamprey Sox genes:** Unrooted phylogenetic tree of Sox genes obtained from analysis performed on MEGA 5.05. The HMG box protein sequences were used in the construction of the tree. Boxes highlight the families of Sox genes. Abbreviations are *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Mm*, *Mus musculus*; *Pm*, *Petromyzon marinus*; *Xl*, *Xenopus laevis*.



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*Chapter 4 - 2***EVOLUTIONARILY CONSERVED ROLE FOR SOXC GENES IN NEURAL  
CREST SPECIFICATION AND NEURONAL DIFFERENTIATION**

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**Abstract**

Members of the Sox family of transcription factors play a variety of critical developmental roles in both vertebrates and invertebrates. Whereas SoxBs and SoxEs are involved in neural and neural crest development, respectively, far less is known about members of the SoxC subfamily. To address this from an evolutionary perspective, we compare expression and function of SoxC genes in neural crest cells and their derivatives in lamprey (*Petromyzon marinus*), a basal vertebrate, to frog (*Xenopus laevis*). Analysis of transcript distribution reveals conservation of lamprey and *X. laevis* SoxC expression in premigratory neural crest, branchial arches, and cranial ganglia. Moreover, morpholino-mediated loss-of-function of selected SoxC family members demonstrates essential roles in aspects of neural crest development in both organisms. The results suggest important and conserved functions of SoxC genes during vertebrate evolution and a particularly critical, previously unrecognized role in early neural crest specification.

## Introduction

Members of the SRY-related high mobility group box (Sox) gene family are expressed in various cell lineages and are critical for cell fate determination and differentiation of many developing tissues (Bowles et al., 2000; Laudet et al., 1993). The Sox family is composed of transcription factors that regulate gene expression through a single high-mobility group (HMG) DNA binding domain (Laudet et al., 1993). The family is divided into ten groups (A–J) based on the HMG sequence and other conserved domains. Sox transcription factors are thought to control transcription by altering chromatin conformation to allow formation of transcription enhancer complexes (Dy et al., 2008; Grosschedl et al., 1994; Kuroda et al., 2005). Because the family members sometime contain trans-activation or trans-repression domains, they are able to interact with different partners to modulate gene expression in a variety of ways.

Sox genes have been shown to play crucial yet distinct roles during neural development. For instance, SoxB proteins Sox1–3 are expressed in neural precursors of the central nervous system, where they function in the maintenance of the progenitor pool population by inhibiting neuronal differentiation (Pevny and Placzek, 2005). Alternatively, SoxEs and SoxDs are critical players in neural crest development (Hong and Saint-Jeannet, 2005). SoxE factors Sox8–10 have been shown to be important for neural crest specification, migration, and diversification in chick (Bell et al., 2000; McKeown et al., 2005), frog (O'Donnell et al., 2006), zebrafish (Yan et al., 2005), and lamprey (McCauley and Bronner-Fraser, 2006). In addition, the SoxD gene LSox5 is also expressed in neural crest progenitors, and cooperates with Sox9 to drive neural crest specification (Perez-Alcala et al., 2004).

The SoxC gene subfamily consists of three single exon genes: Sox4, Sox11, and Sox12 (also known as Sox22). Although these genes are best known for their roles in cancer (Ikushima et al., 2009; Penzo-Mendez, 2010; Vegliante et al., 2013; Wasik et al., 2013), past studies have examined the effects of SoxC genes in neural development. Sox4, Sox11, and Sox12 are all expressed in the post-mitotic neurons of the central nervous system (Cheung et al., 2000; Dy et al., 2008) in a pattern that is complementary to SoxBs. SoxCs seem to be important for neuronal differentiation, driving expression of pan-neuronal proteins (Bergsland et al., 2006). This is consistent with data obtained from mouse knockout studies, which reveal a decrease in neuronal survival and loss of differentiated neurons (Bhattaram et al., 2010). Recently, Sox4 and Sox11 also have been shown to be crucial for survival of sympathetic ganglia (Pötzner et al., 2010) and sensory neurons of the trigeminal and dorsal root ganglia (Lin et al., 2011). Thus, SoxCs seem to have an important role in development of neuronal derivatives of the neural crest.

While these results highlight a later role of SoxCs in neural crest diversification, little is known about the involvement of these factors in the early steps of neural crest formation. Similarly, there is no information regarding the expression or function of SoxCs in development of basal vertebrates, making it difficult to infer the pan-vertebrate role of this gene family. Although the expression pattern of other major Sox gene subfamilies have been described in the sea lamprey (*Petromyzon marinus*) (Uy et al., 2012), here we expand this analysis to include the SoxC subfamily and characterize the function of the lamprey SoxC orthologs in neural crest formation in a basal vertebrate. To this end, we have cloned and characterized the expression patterns and function of the lamprey SoxC gene family, termed SoxC1–C4, during neural crest development. To assess conservation of the roles of

SoxCs in vertebrate development, we compared these to their homologs (Sox4, Sox11, and Sox12) in *Xenopus laevis*. We find that, along with the *X. laevis*, SoxCs, SoxC1, SoxC3, and SoxC4 are expressed in neural crest cells and/or their derivatives and play an important role in aspects of their development. Intriguingly, the results reveal a previously unknown early function for SoxC genes in neural crest specification that is conserved between lamprey and *X. laevis*.

## **Methods and materials**

### ***Cloning and characterization of SoxC genes***

We used a variety of methods to clone the SoxC genes from frog (*X. laevis*) and lamprey (*P. marinus*). The full-length clones of *X. laevis* Sox4 and Sox11 genes were obtained commercially through Open Biosystems while *X. laevis* Sox12 was PCR amplified from a cDNA library and TOPO cloned. Obtention of full-length lamprey SoxCs required a combination of heterospecific macroarray screening and 5' RACE as described below.

### ***Heterospecific screening of an arrayed lamprey embryonic cDNA library***

A high-quality directional full-length arrayed cDNA library (Sauka-Spengler et al., 2008) from embryonic day 2–12 lamprey embryos was used for low-stringency screening. Nine nitrocellulose filters were screened using Sox heterospecific probes from *Gallus gallus* SoxC genes, yielding four different SoxC genes whose identities were confirmed by sequencing on both strands and by BLAST searching the database. The SoxC1 and SoxC4

clones were full length while SoxC2 and SoxC3 were truncated and thus required 5' RACE for the completion of the mRNA sequence.

### ***RNA-ligated mediated 5' rapid amplification of cDNA ends (RLM-5' RACE)***

Total RNA was extracted from 6, 8, 10, and 14 days-old embryos using the Ambion RNAqueous kit. RLM-5' RACE was conducted on the total mRNA with the Invitrogen GeneRacer Kit. Total RNA was dephosphorylated through Calf Intestinal Phosphatase (CIP) treatment, decapped via Tobacco Acid Pyrophosphatase (TAP), ligated with the GeneRacer RNA oligo, and finally reverse-transcribed using random hexamer priming to form the cDNA template. SoxC fragments were amplified by touchdown PCR according to the Invitrogen GeneRacer Kit. The gene specific primers used were SoxC2: 5' ACGACGGGACGGATGACAAAGCA 3' and SoxC3: 5' GGGTGGCCCTCTCGCTTGCTC 3'.

### ***Cloning the PCR product***

Gel extraction of the PCR product was conducted according to the Qiagen:QIAquick Gel Extraction Kit protocol and cloned with Invitrogen: TOPO TA Cloning. The clones were selected against the metabolism of X-gal and the production of  $\beta$ -galactosidase, purified following the QIAprep spin miniprep kit, and sent for sequencing. (Davis Sequencing, Davis, CA).

### ***Embryo collection and maintenance***

Mature or maturing *P. marinus* adults were obtained from Hammond Bay Biological Station, Millersburg, MI, USA. Mature animals were maintained in our lamprey facility in

12 °C chilled re-circulating water and used for spawning. Juveniles were kept at lower temperatures before the onset of maturation and then progressively induced to maturation by gradually augmenting the water temperature and expanding the daylight cycle. For in vitro fertilization, eggs were stripped manually from a single gravid female into a 500 ml crystallizing dish containing 100–200 ml of spring water, and milt from a spermiated male is then applied directly onto the eggs. After 15 min, the fertilized eggs are washed through several changes of distilled 18 °C water and placed in spring water (Sparkletts Water) in the 18 °C incubator. After the first division, the embryos were transferred to 0.1X MMR (Marc's Modified Ringers) for long-term culture. The medium from each culture is replaced with fresh ringers every day to avoid fungal infection. Embryos were fixed in MEMFA (4% formaldehyde, 0.1 M MOPS (pH 7.4), 1 mM MgSO<sub>4</sub>, 2 mM EGTA), dehydrated gradually, and stored in 100% methanol at 20 °C (Sauka-Spengler et al. 2007).

### ***Morpholino knockdown***

FITC-labeled morpholino antisense oligonucleotides (Gene Tools, Philomath, OR, USA) were designed to target the translation initiation site. The sequences of the oligonucleotides used are:

5'-AACGAGCTTATGGTCAGTAACAATA w/FITC-3' Control MO.

5'-TGGCCGTCCTGCGAGTTCCACGCTC w/FITC-3' SoxC1 MO.

5'-CGTGCAGCACCATGCTCGCTCAGCC w/FITC-3' SoxC3 MO.

5'-ATCGTGGATCGCCCGCTGCCTCCGA w/FITC-3' SoxC4 MO.

5'-TGTTGTTGGTTTGTTCACCATTC w/FITC-3' XI Sox4a/b MO.

5'-TGTCCATGTCTGCTCGCTGCACCAT w/FITC-3' XI Sox11a/b MO.



5'-CCCGTAGTCTTGTCTGCACCATCC w/FITC-3' XI Sox12 MO.

For lamprey embryo injections, approximately 10 ng of morpholino was injected into a single blastomere at the two-cell stage as described (Sauka-Spengler et al., 2007). Embryos were raised until the desired stages, fixed, and dehydrated as described above. *Xenopus* embryos were injected with 10 nL of morpholino at 0.6 M with 30 ng/uL of lacZ at the two cell stage. For rescue experiments, we co-injected morpholinos with their respective SoxC mRNA containing mutations in the morpholino binding site. SoxC mRNAs were introduced at 25 ng/uL in lamprey and 40 ng/uL in *Xenopus*. mRNAs were synthesized using mMessage mMachine SP6 transcription kit (Life technologies).

***β-gal lineage tracing, in situ hybridization, and histology***

*X. laevis* embryos were injected with 0.3 ng of lacZ mRNA for lineage tracing. Embryos were fixed for 30 min in MEMFA, developed in 5 mM ferricyanide, 5 mM ferrocyanide, 1 mg/mL X-gal, and 2 mM MgCl<sub>2</sub> solution at 37 °C for 15 min, and re-fixed for 1 h at room temperature.

Whole-mount in situ hybridization in lamprey and *X. laevis* embryos was performed using digoxigenin or RNA probes according to Xu and Wilkinson (1998) with the following modifications: prior to treatment with Proteinase K, embryos were incubated in the bleaching solution (0.5 SSC, 5% formamide, 10% H<sub>2</sub>O<sub>2</sub>), according to Broadbent and Reed (Broadbent and Read, 1999) under direct light for 10–15 min. The concentration and the length of Proteinase K treatment (20 µg/mL, 10 min) was constant for embryos of all stages. Hybridization and subsequent washes were carried out at 70 °C in hybridization solution

containing 50% formamide, 1.3 SSC, 5 mM EDTA pH8.0, 200 µg/mL yeast tRNA, 100 µg/ml heparin, 0.2% Tween-20, and 0.5% CHAPS. The hybridization signal was detected using BM Purple substrate (Roche, Indianapolis, IN) for early-stage embryos (E3–E10) or NBT/BCIP (Roche, Indianapolis, IN) for later stages. After photographing, embryos were post-fixed in 4% paraformaldehyde/PBS, rinsed in PBS, cryo-protected in two subsequent steps (15% sucrose/PBS and 7.5% gelatin/15% sucrose/PBS, equilibrated and mounted in 20% gelatin/PBS), and frozen with liquid nitrogen. 10 µm cryosections were collected on Super Frost Plus slides (Fischer Scientific, Pittsburgh, PA) (Sauka-Spengler et al., 2007).

### ***Morpholino efficiency assay***

To test for morpholino efficiency, we assembled GFP fusion constructs of each of the SoxC genes. These constructs contained 100 bp of the 5'UTR and the first 30 bp of the coding region fused to the GFP coding sequence, thus encompassing the morpholino target site.

*X. laevis* embryos were injected as described (Sauka-Spengler et al., 2007) with the mRNA of each SoxC gene fusion construct and each one of the morpholinos used. Constructs were injected at 100 pg along with either control or targeted morpholinos at the same concentrations as used for their respective experiments. Efficiency of the morpholinos was confirmed by absence of GFP protein. Wholemount immunostaining was conducted with antiGFP rabbit IgG from Invitrogen at (1:200).

### ***Phylogenetic analysis***

The amino acid alignments and Neighbor Joining (NJ) tree were constructed using ClustalX. The phylogenetic trees were visualized using Tree View v. 0.5.0.

Protein sequences from other species retrieved from GenBank carry the following nomenclature abbreviations: Am, *Ambyostoma mexicanum*; Bb, *Branchostoma belcheri*; Bf, *Branchiostoma floridae*; Ci, *Ciona intestinalis*; Cs, *Ciona savignii*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Hr, *Halocynthia roretzi*; Lv, *Lytechinus variegatus*; Md, *Monodelphis domestica*; Mm, *Mus musculus*; Pl, *Paracentrotus lividus*; Pm, *Petromyzon marinus*; Sc, *Scyliorhinus canicula*; St, *Scyliorhinus torazame*; Sp, *Strongylocentrotus purpuratus*; Xl, *Xenopus laevis*; Xt, *Xenopus tropicalis*.

### ***Embryo phenotype scoring***

*X. laevis* and lamprey embryos exhibiting a phenotype were scored at stage 25 and E8–10, respectively, by a strong phenotype (loss of one or all late stage structures), moderate phenotype (exhibiting a difference in expression level), or no phenotype. Based on these numbers, statistical analysis was conducted using contingency tables (<http://www.physics.csbsju.edu/stats/>) to obtain a chisquared value.

## Results

### *Overlapping expression of the SoxC family genes in lamprey embryos*

We identified orthologs of the SoxC family genes in lamprey by conducting heterospecific screenings of a macroarrayed cDNA library (Sauka-Spengler et al., 2007), conducting bioinformatic analysis on the lamprey genome (Smith et al., 2013), and using rapid amplification of cDNA ends (5' RACE). Four orthologs were identified in *P. marinus*: SoxC1–C4 (Fig. S1). Using *in situ* hybridization, we examined the distribution patterns of SoxC family members from gastrulation through early organogenesis, corresponding to embryonic days (E) 4–14, with emphasis on expression in the neural crest and its derivatives. Our results show that the lamprey SoxC genes SoxC1, SoxC3, and SoxC4 are expressed in the neural plate border and migrating neural crest, as well as in some neural crest derived ganglia. We observed a high degree of overlap between the expression domains of the paralogs in the neural tube, cranial ganglia, and branchial arches.

Onset of SoxC1 initiates at low levels during gastrulation in the neural plate border and posterior mesoderm at E4 (Fig. 1A and Q), but increases by E5 in the dorsal neural tube where the neural crest progenitors are located (Fig. 1B and T). Sections reveal that SoxC1 is expressed in the neural tube, trigeminal and epibranchial ganglia, and endostyle (Fig. S2D', D'', and E'). Later, it is expressed in the neural crest derived and mesenchymal portions of the branchial arches, where it persists through E14, the last stage examined (Fig. S2F, F' and F''). Unlike the other

paralogs, SoxC2 is barely detectable at the neural plate and neural plate border at stage E4. Transcripts are detected at E5 in the closing neural plate (Fig. 1F) and neural tube at stage E6 (Fig. 1G). In addition to the neural tube, SoxC2 is widely expressed in the notochord, otic vesicle, trigeminal ganglia, and endoderm derived portions of the branchial arches (Fig. S2L, L and L''').

Like SoxC1, SoxC3 is also expressed in the neural plate and neural plate border at E4 (Fig. 1I and R), prominently in the neural folds at E5 (Fig. 1J) dorsal neural tube at E6 (Fig. 1G and V), and in migrating neural crest cells (arrow in Fig. 1V). It is very strongly expressed in the dorsal portion of the neural tube and in the lateral portions of the branchial arches at E8 (Fig. 1L and Fig. S2P, P', P'') corresponding to the ectoderm and neural crest. At E10, the otic vesicle and the branchial arches begin to express significant levels of SoxC3 (Fig. S2Q and Q'''). At E14, SoxC3 is highly expressed in branchial arch-derived cartilage (Fig. S2R and R'). Finally, the expression domain of SoxC4 at E4 is broad, but enhanced expression is detected in the neural plate and neural plate border (Fig. 1M and S). At E6, SoxC4 transcripts are detected in the neural tube, ectoderm, and endoderm layers (Fig. 1V and W). SoxC4 is strongly expressed in migrating cranial neural crest cells of early embryos and in the branchial arches, particularly in the neural crest domain and mesodermal core of older embryos (Fig. S2W'' and X'''). In addition to the neural crest, it is expressed in the ectoderm and somites (Fig. 1P and Fig. S2T' and V'), optic pit, and vesicle (Fig. S2W', X', and X'').

### ***Expression of Sox4, Sox11, and Sox12 in Xenopus laevis***

Using *in situ* hybridization, we examined the distribution patterns of SoxC family members in *X. laevis* from gastrulation through early organogenesis (stages 14–26), focusing on expression in the neural crest and derivatives. Similar to lamprey and mouse (Dy et al., 2008), there is large overlap between the expression patterns of Sox4, 11, and 12 in *X. laevis*. At approximately stage 14, Sox4, Sox11, and Sox12 are expressed broadly throughout the neural plate and later in migrating neural crest through stage 20 (Fig. 2A–C, F–H, and K–M). Expression of Sox4 and Sox11 is very widespread with transcripts detected in developing cranial ganglia and branchial arches. Elevated levels of expression in forming ganglia and branchial arches can be observed at stage 26 (Fig. 2D, I, and N). Later, the SoxCs are expressed in the otic vesicle, the lens, neural tube, and branchial arches. Expression of Sox4 and Sox12 appears to be fairly ubiquitous in the head (Fig. 2E, J, and O). Thus, lamprey and *X. laevis* SoxC orthologs have similar expression domains, especially with respect to the neural crest and its derivatives.

### ***Early role of the SoxCs in the neural crest***

Expression of SoxC paralogs in the neural plate border of both lamprey and *X. laevis* embryos suggests a possible role for these genes in the specification of the neural crest. To investigate this possibility, we examined the early function of the SoxCs using morpholino-mediated gene knockdown in both model organisms. Phenotypic analysis focused on effects on early neural crest marker genes. For lamprey, we designed morpholinos targeted to SoxC1, C3, and C4, since they were

the paralogs expressed in the neural plate border, neural crest, and its derivatives. The morpholinos for *X. laevis* Sox4 and Sox11 were designed to eliminate both isoforms of the gene Sox4a/b and Sox11a/b, respectively.

To test efficiency, we performed a control experiment in which either blocking morpholino or control morpholino was injected together with mRNA encoding ~ 100 bp of the 5' UTR plus 10 amino acids of the target proteins fused to GFP into one cell stage frog embryos. The results show that function blocking morpholinos, but not control morpholinos, specifically knock-down GFP translation of their respective target construct, demonstrating that they efficiently eliminate production of the target protein (Fig. 3). Furthermore, the morpholinos do not affect translation of other SoxC–GFP fusion constructs that lack their full target sites (data not shown), highlighting their specificity.

We next examined the effects of morpholinos targeting SoxC genes in lamprey. To this end, a single blastomere of two-cell embryos was injected with the morpholino, and embryos were cultured until early stages of neural crest specification (E6), when they were fixed and processed for *in situ* hybridization. Lamprey embryos injected with SoxC1, C3, or C4 morpholino exhibited down-regulation of bona fide neural crest marker SoxE1 and FoxD3 on the injected side compared with the control side (Fig. 4A–F), as revealed by *in situ* hybridization. In contrast, there was no significant effect on embryos injected with control morpholino (Fig. 4J and K). Importantly, rescue experiments in which morpholinos

were co-injected with mRNAs encoding their target protein, with mutations in the morpholino binding site, resulted in expression of the neural crest specifier gene *FoxD3* at near normal levels, further confirming specificity of the morpholinos (Fig. 4G–L). These results support an important role for SoxCs in neural crest specification in the lamprey.

To assess whether the role of SoxCs in neural crest specification is conserved in other vertebrates, we performed similar loss-of-function experiments in *X. laevis* and examined the effects of SoxC loss of function at stage 14. Similar to lamprey, knockdown of Sox4a/b and Sox11a/b in *X. laevis* embryos results in loss of neural crest specifiers *FoxD3*, *Sox10*, and *Twist* (Fig. 5A–F and I–K). To demonstrate specificity, we performed rescue experiments in which morpholinos were co-injected with mRNA encoding their target protein. The results reveal very good rescue of neural crest specifier gene expression, further confirming specificity of the morpholinos (Fig. 5G, H, and L). Embryos injected with Sox12 MO were not viable and did not develop to neurulation (data not shown). Taken together, our data reveal a previously unknown role for some SoxC genes in the process of neural crest formation that appears to be conserved between gnathostomes and basal vertebrates.

### ***Loss of SoxC genes leads to a loss of neuronal fate in lamprey***

SoxCs play an important role in the establishment of sympathetic, trigeminal, and dorsal root ganglia in amniotes. To assess if this is evolutionarily conserved, we investigated the role of SoxC genes in later stages of neural crest development.



Given that SoxC1, SoxC3, and SoxC4 are expressed not only in premigratory but also migratory and neural crest-derived tissue, we examined the later effects of morpholino-mediated knockdown on the morphogenesis of neural crest derived ganglia. In lamprey, morpholinos (MO) targeted to SoxC1, SoxC3, and SoxC4, and in *X. laevis* Sox4 and Sox11, were injected into one blastomere of the two-cell stage embryo. We then we examined the subsequent effects on ganglion formation using *in situ* hybridization with neurogenin as a marker for neuronal differentiation at E8–10 in lamprey and stage 26 in *X. laevis*.

In E8.5 lamprey embryos, the number of neurogenin positive cells in the neural tube, acoustic ganglion, epibranchial ganglia, and dorsal root ganglia was greatly diminished after SoxC1 knockdown (Fig. 6A and B) when compared to the uninjected side (Fig. 6C). At E10 from a dorsal view, we observed a strong effect of the SoxC1 morpholino on the formation of the dorsal root ganglia and epibranchial ganglia (Fig. 6D–F) compared to the normal reiterative pattern of ganglia seen on the control side. Similar effects were seen after knockdown of SoxC3. At E8, neurogenin expression was down-regulated on the injected side in the cranial ganglia and forming branchial arches (Fig. 6G–I), and by E9, neurogenin was down-regulated in the acoustic ganglion and epibranchial ganglia (Fig. 6J–L). Similarly, SoxC4 loss-of-function drastically affects morphogenesis of the acoustic, trigeminal, and epibranchial ganglia. These structures are completely lost on the injected side at E8.5 (Fig. 6M–O) and E10 (Fig. 6P–R). Interestingly, neural tube expression of neurogenin was not affected by the SoxC4 knockdown.

Knockdown of SoxCs in *X. laevis* also resulted in strong effects on the formation of neural crest-derived structures. Sox4MO resulted in loss of neurogenin in a variety of structures including the branchial arches, otic vesicle, and optic lens (Fig. 7A–C).

Sox11MO produced similar results (Fig. 7D–F) with the loss of neurogenin expression in the epibranchial ganglia and otic vesicle. Over-expression of the XlSoxC genes resulted in increased Ngn-2 expression, which is a known bHLH factor downstream of the SoxCs. Taken together, our results point to an evolutionarily conserved dual role of SoxCs in the formation of neural crest-derived ganglia. First, SoxCs appear to be important for neural crest specification as shown by the loss of the neural crest specifiers SoxE1/Sox10 and FoxD3 in morphant embryos. This is a novel role for SoxC genes that has not been described previously. Second, our results reveal a requirement for SoxCs genes in the survival of neural crest-derived ganglia (Lin et al., 2011) that is conserved in lamprey and *X. laevis*, highlighting that this later role of SoxCs in neural crest development is shared with basal vertebrates.

## Discussion

During development, SoxC genes are best known for their roles in neural precursor proliferation and neuronal survival in the central and sympathetic nervous system (Bhattaram et al., 2010; Cheung et al., 2000; Hoser et al., 2008; Potzner et al., 2010). In mice, SoxCs are functionally redundant, with double knockouts resulting in loss of differentiated neurons (Hoser et al., 2008) and heart defects (Hong and Saint-Jeannet, 2005). Triple Sox4/11/12

knockout animals die at mid-gestation with massive death of neural and mesenchymal progenitors (Bhattaram et al., 2010). SoxC genes are thought to act in conjunction with SoxBs during neuronal differentiation. SoxBs are expressed in neural precursors and function in maintaining those cells in a progenitor state. As neural precursors enter the post-mitotic differentiation program, they start expressing SoxC genes, which drive expression of proteins involved in neural fate commitment (Bergsland et al., 2011, 2006; Cheung et al., 2000; Hoser et al., 2008). SoxCs also have been shown to play key roles in the development of neural crest-derived ganglia. Sox4/11 knockout mice exhibit defective proliferation and survival in the sympathoadrenal lineage (Pötzner et al., 2010). Additionally, Sox11 knockout mice have a marked reduction in sensory neuron survival in the trigeminal and sensory ganglia (Lin et al., 2011). However, the role of SoxCs in early stages of neural development had not been investigated in depth.

In the present study, we describe a novel and evolutionarily conserved role for SoxC genes in neural crest development. We find that lamprey SoxC1–C4 family members exhibit a high degree of similarity in their expression patterns in relation to amniotes (Dy et al., 2008). In chicken and mouse, Sox4 and Sox11 are expressed in the pharyngeal arches, heart, and various ganglia, similar to lamprey SoxCs (de Martino et al., 2000; Hong and SaintJeannet, 2005; Maschhoff et al., 2003; Uwanogho et al., 1995). In both lamprey and *X. laevis*, we observed widespread expression of SoxC genes in the neural plate and neural tube. During later embryogenic development, SoxC genes are co-expressed in cranial ganglia and branchial arches. More importantly, most of the SoxC genes were expressed in the neural

plate border, a finding which suggested a potential role for these transcription factors in neural plate and/or neural crest specification.

Consistent with this possibility, our results reveal that loss of lamprey SoxC1, SoxC3, and SoxC4 yield similar phenotypes, all resulting in down-regulation of the neural crest specifier genes SoxE1 and FoxD3 (Fig. 4). This role is conserved in *X. laevis*, as Sox4 and Sox11 knockdowns in this species also diminish expression of neural crest specifier genes Sox10, FoxD3, and Twist (Fig. 5). In addition, knockdown of SoxCs results in defects of neural crest-derived ganglia in both *X. laevis* and lamprey after neurulation (Fig. 6 and 7). One possible interpretation of these results is that the later knockdown effects in ganglia formation are secondary to effects at the neural plate border or closing neural tube that lead to abnormal neural crest specification. However, data in other model organisms suggest otherwise. Bhattaram et al. (2010) have recently identified the transcription factor Tead2 as a direct target of SoxC in neural progenitors in the mouse. Tead2 is a transcriptional mediator of the Hippo signaling pathway, and it seems to play an important part in controlling survival of neural and mesenchymal progenitors during embryonic development (Bhattaram et al., 2010). Our results in lamprey and *X. laevis* highlight an early role for SoxCs, at the neural plate border during neural crest specification, rather than changes in cell survival and proliferation. Thus, it is likely that SoxC genes may play multiple developmental roles in vertebrates, first acting in neural crest commitment at the neural plate and later ensuring progenitor cell survival in the neuronal lineages.

Studies scrutinizing the role of SoxCs in disease may provide important clues regarding the mechanisms through which these transcription factors drive neural crest specification. In addition to various roles in metazoan development and organogenesis, SoxC genes play critical roles in many types of cancer (Wasik et al., 2013). For example, Sox4 expression level is elevated in glioma, breast, prostate, small cell lung, and medulla blastoma cancer types. In vitro studies have shown that Sox4 is a direct target of TGF- $\beta$  signaling and functions as an oncogene to maintain stemness of gliomas (Ikushima et al., 2009). Sox4 is known to stabilize  $\beta$ -catenin to activate TGF- $\beta$  signaling, thereby maintaining Wnt signal transduction and play a role in the epithelial to mesenchymal transition in vitro (Vervoort et al., 2013; Zhang et al., 2012). The role of Wnt signaling in neural crest specification is well known (Garcia-Castro et al., 2002; Sauka-Spengler and Bronner-Fraser, 2008), and thus it is possible that SoxCs could be cooperating with this signaling pathway in instructing cells of the neural plate border to adopt a neural crest fate.

It has been suggested SoxCs have redundant roles and even compete with one another for binding to their targets due to their similar spatial and temporal expression and conserved HMG domains (Dy et al., 2008). Surprisingly, while SoxCs have been shown to have a large degree of functional redundancy in mouse (Bhattaram et al., 2010), in lamprey we found that knockdown of each of the three lamprey orthologs was sufficient to affect both neural crest specification as well as neuronal survival in neural crest-derived ganglia. This indicates that lamprey SoxCs have lower functional redundancy than their amniote counterparts. Indeed, while lamprey SoxCs genes have similar expression patterns, we also observed expression domains unique to certain paralogs. For instance, SoxCs are found in

either the mesenchymal or ectodermal portions of the branchial arches and various cranial ganglia, sometimes in different sub-regions of these structures (Figs. 1 and S2). Such differences may result from divergence of function accompanying independent gene duplication in jawless vertebrates (Green and Bronner, 2013).

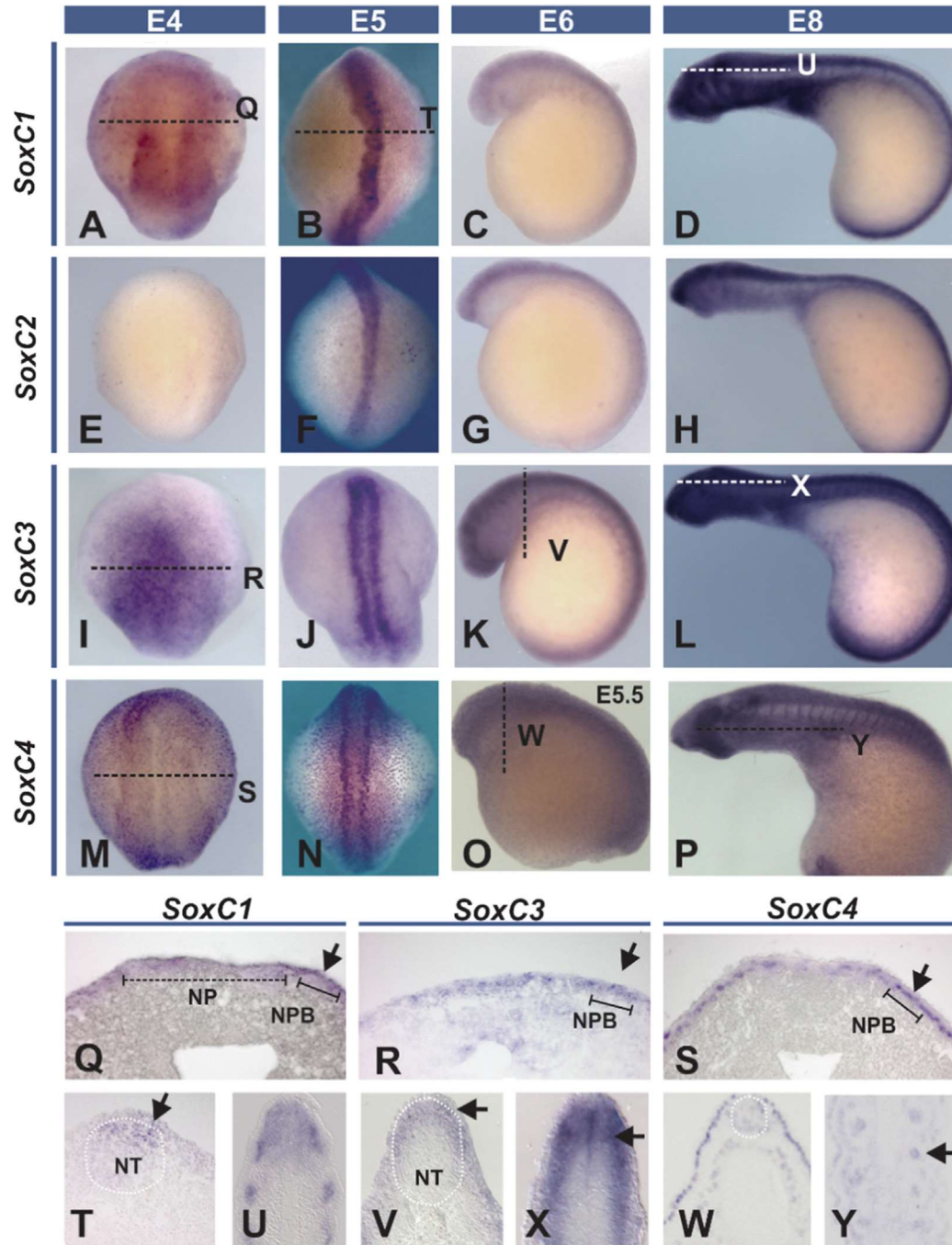
Our results also point to a possible role of SoxCs in the evolution of the vertebrate neural crest. In contrast to the multiple family members observed in vertebrates, there is a single copy of SoxC in invertebrates such as *Drosophila*, *Amphioxus*, *C. elegans*, and ascidians (Cremazy et al., 2001). The *Drosophila* SoxC gene is a dual exon gene involved in mesoderm survival and dendritic pruning (Kirilly et al., 2009; Osterloh and Freeman, 2009). The single *Amphioxus* SoxC gene exhibits a broad expression pattern similar to the cumulative pattern of vertebrates Sox4, Sox11, and Sox12 (Lin et al., 2009). During gastrulation and neurulation, *AmphiSoxC* is expressed in the dorsal ectoderm, mostly in the neural ectoderm, and endomesoderm fates (Lin et al., 2009). The finding that lamprey has four SoxC orthologs indicates that the origin of neural crest cells in vertebrates coincided with more than one round of duplication of SoxC genes. Given the conserved role of SoxCs in multiple stages of neural crest development, it is tempting to speculate that duplications in the SoxC gene family may have contributed to the evolution of neural crest in vertebrates and the elaboration of the vertebrate peripheral nervous system.

**Acknowledgment**

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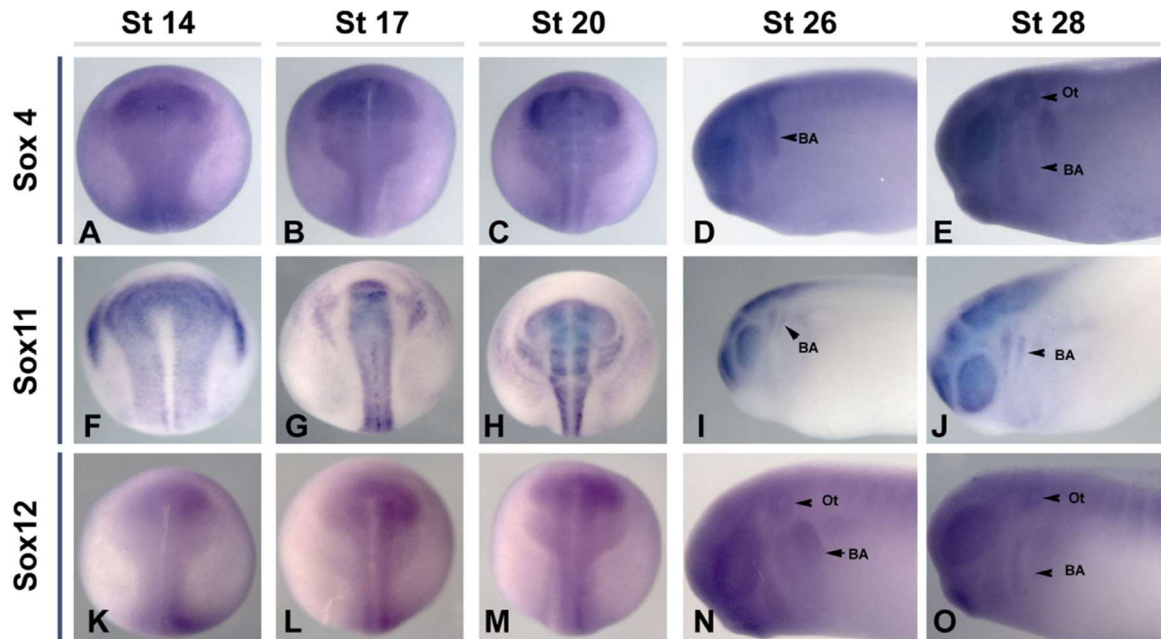
**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2014.09.022>.

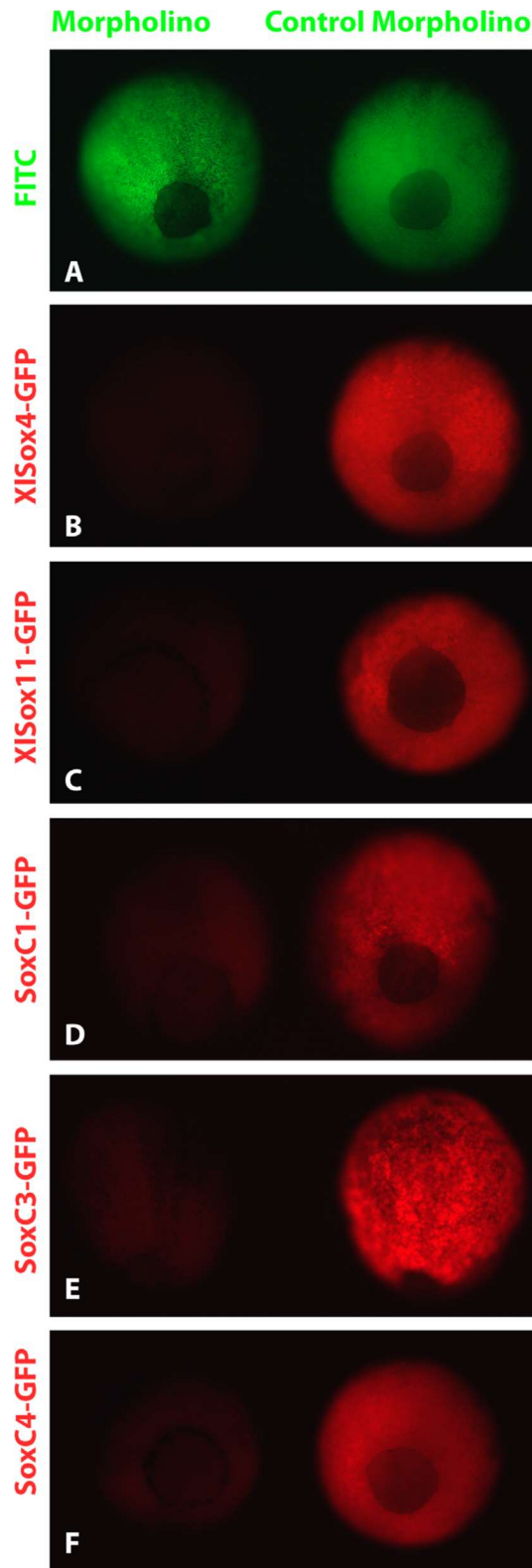


**Fig. 1. SoxC1–C4 are expressed in the neural plate border, neural tube, and neural crest derivatives in lamprey.** Expression patterns of SoxC1 (A–D), SoxC2 (E–H), SoxC3 (I–L), and SoxC4 (M–P) in lamprey embryos from day (E) 4 to day 8 of development. SoxC1 (Q), SoxC3 (R), and SoxC4 (S) are expressed in the forming neural plate and neural plate border while SoxC2 transcript was not detected at the gastrula stage (E). (T–Y) Cross sections of embryos (A–P), showing SoxC expression in the dorsal neural tube (T and V), neural tube (U–W), and neural crest derivatives (U and Y). NT: neural tube, NP: neural plate, NPB: neural plate border.



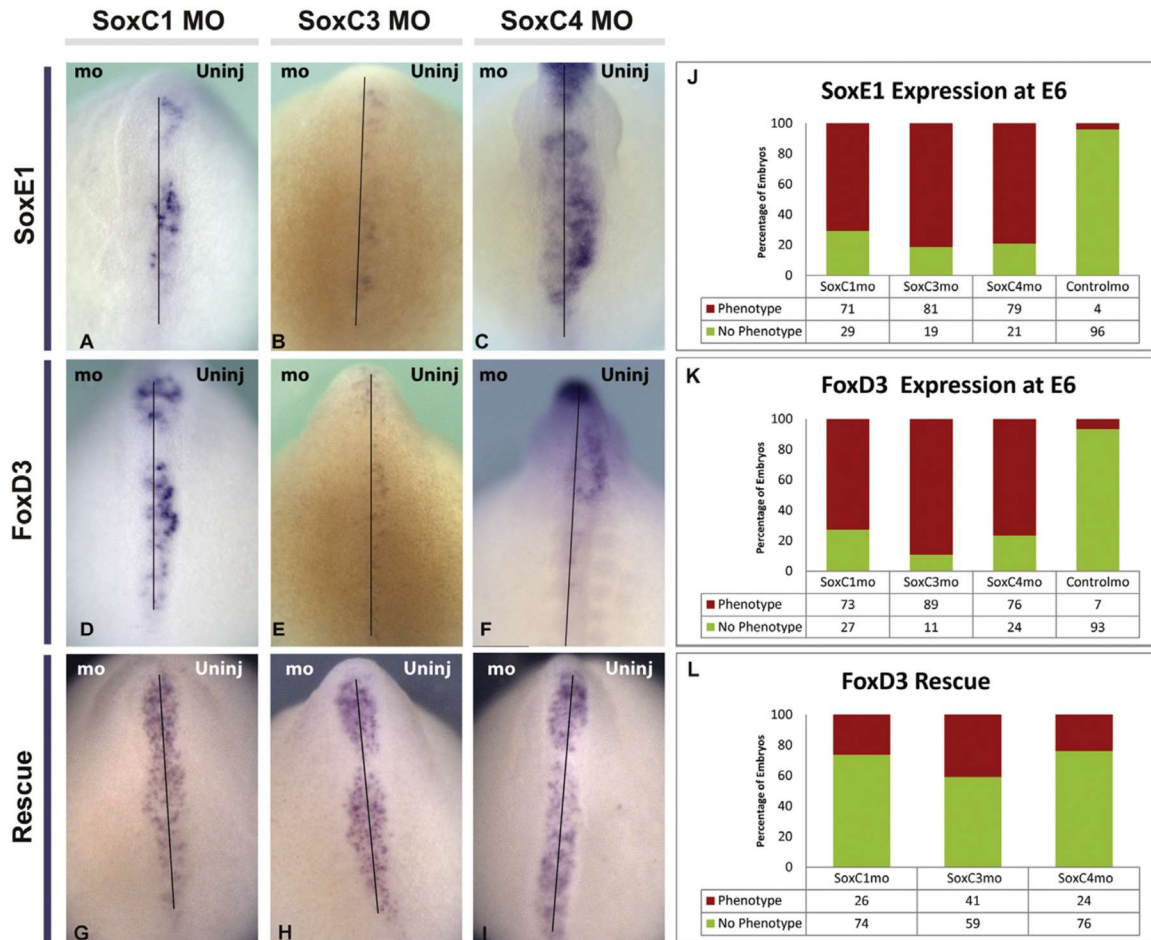


**Fig. 2. *Xenopus laevis* SoxCs exhibit overlapping expression in the neural plate, neural crest, and facial region.** Sox4 (St.14–28) is expressed broadly in the neural plate starting at stage 14/15 and in the forming neural tube and migrating neural crest (Dorsal: A–C). It is then strongly expressed in developing ganglia as well as weakly in the branchial arches, otic vesicle, and eye (D and E). Sox11 (St.14–28) is strongly expressed early in the neural plate border and neural folds (Dorsal:H). It is expressed in neural crest and ectodermal placodes (Dorsal:F–J). Expression is maintained in the neural tube, forming ganglia, and branchial arches (I and J). Sox12 (st.14–28) is expressed broadly and similarly to Sox4. At St 14–20, Sox12 is expressed throughout the neural plate and in the migrating neural crest (Dorsal:K–M). Expression is observed throughout the neural tube as well as in the cranial ganglia, branchial arches, eye, and otic vesicle (N and O). Ot: Otic Vesicle; BA: Branchial Arches.

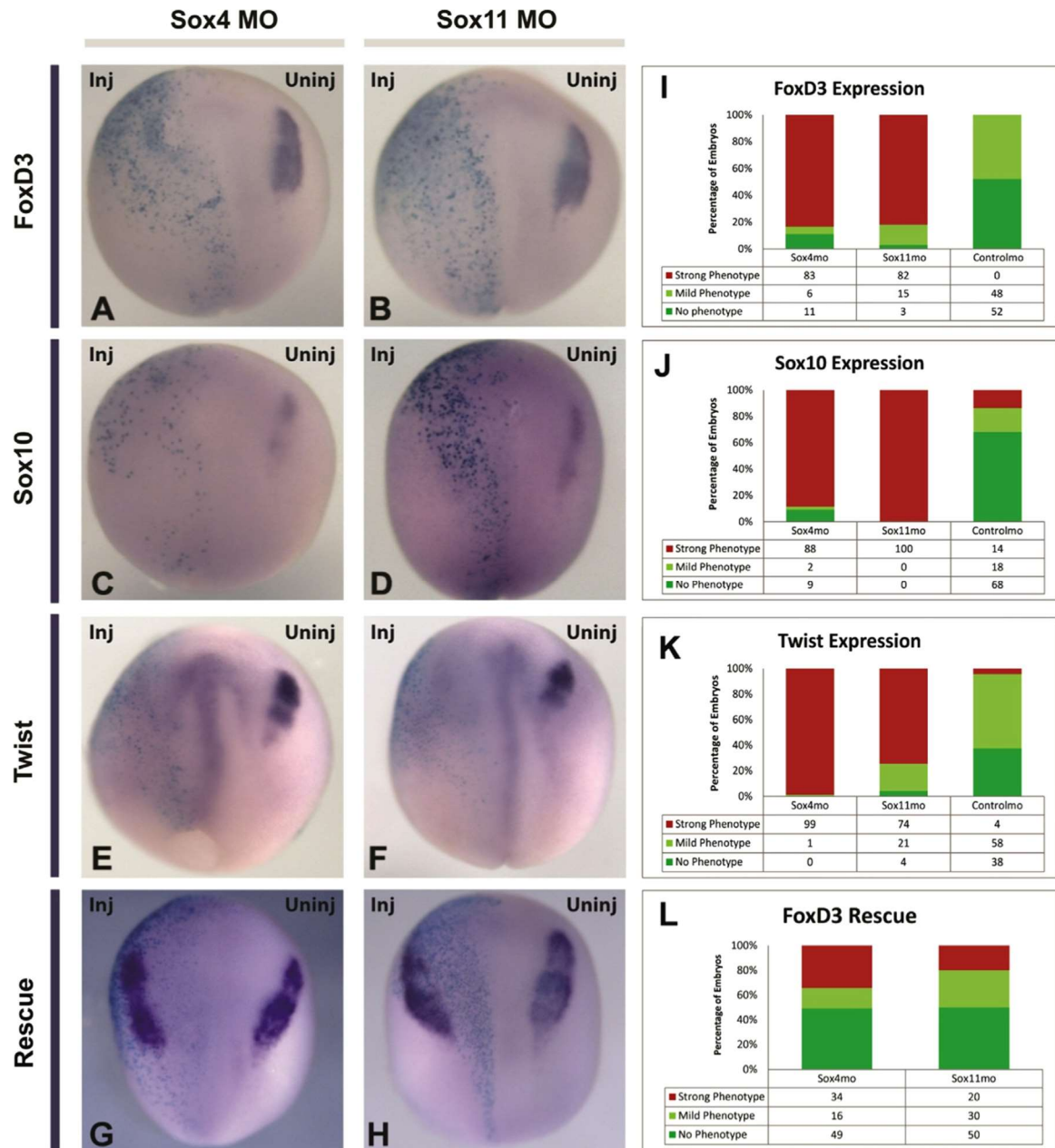


**Fig. 3. Morpholino antisense oligonucleotides efficiently disrupt target protein translation.**

Side by side comparison of *Xenopus laevis* embryos injected with 5'UTR-GFP mRNA (left column) and targeted morpholino versus 5'UTR-GFP mRNA and control morpholino (right column). (A) Embryos injected with either control or targeted FITC labeled morpholinos. (B–F) *X. laevis* embryos stained with anti-GFP antibody show abundance of GFP in embryos injected with control morpholino, while targeted morpholino efficiently prevents mRNA translation and GFP protein production.



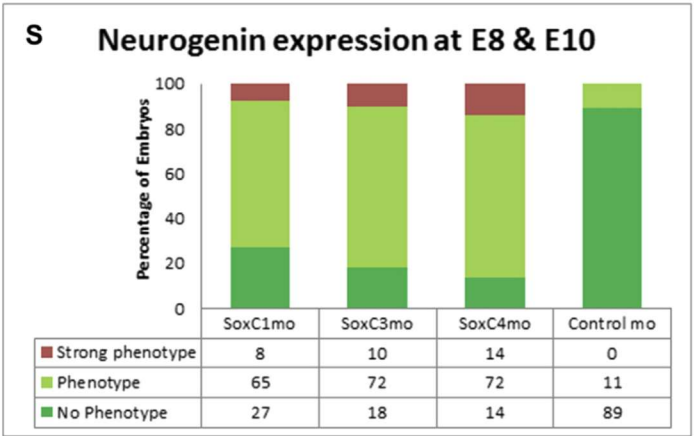
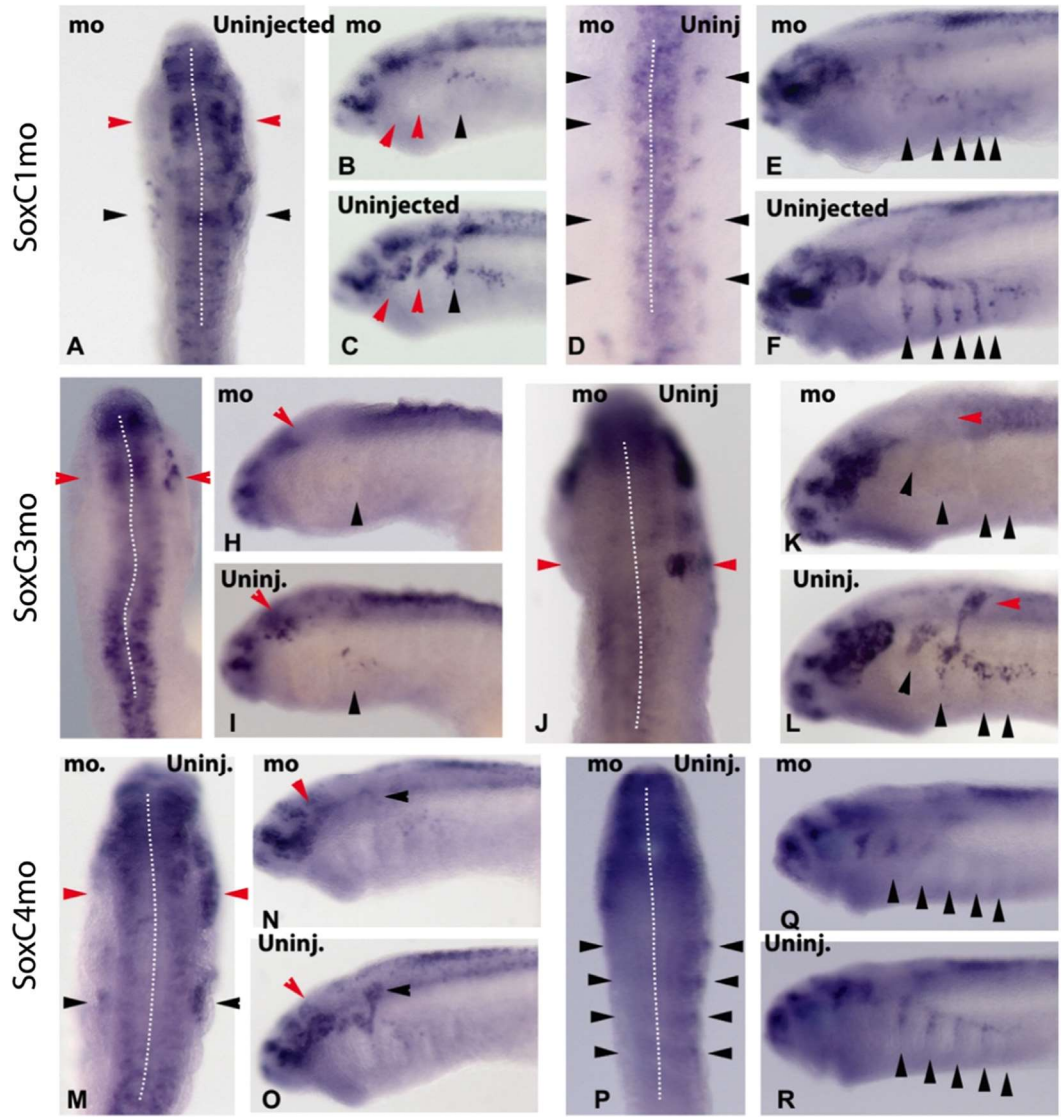
**Fig. 4. Loss of SoxC1, C3, and C4 in lamprey results in loss of neural crest specifier genes Sox10 and FoxD3:** AtE6: Knockdown of SoxC1 (A and D) results in a loss of expression of SoxE1 and FoxD3. Loss of SoxC3 (B and E) causes loss of expression in SoxE1 and FoxD3. Loss of SoxC4 results in loss of expression in SoxE1 and FoxD3 (C and F). (J) Quantification of effects with SoxE1: SoxC1mo(n=24), SoxC3mo(n=43), SoxC4mo(n=38), and Controlmo(n=26) ( $\chi^2=50.2$  with 3 degrees of freedom). (K) FoxD3: SoxC1mo(n=22), SoxC3mo(n=37), SoxC4mo(n=68), and Controlmo(n=76) ( $\chi^2=103$  with 3 degrees of freedom). (G–H) SoxCmo injected embryos rescued with SoxC mRNA by FoxD3 expression. (L) Quantification of effects with FoxD3: SoxC1mo(n=23), SoxC3mo(n=27), and SoxC4mo(n=21); ( $\chi^2=1.97$ ) with 2 degrees of freedom.



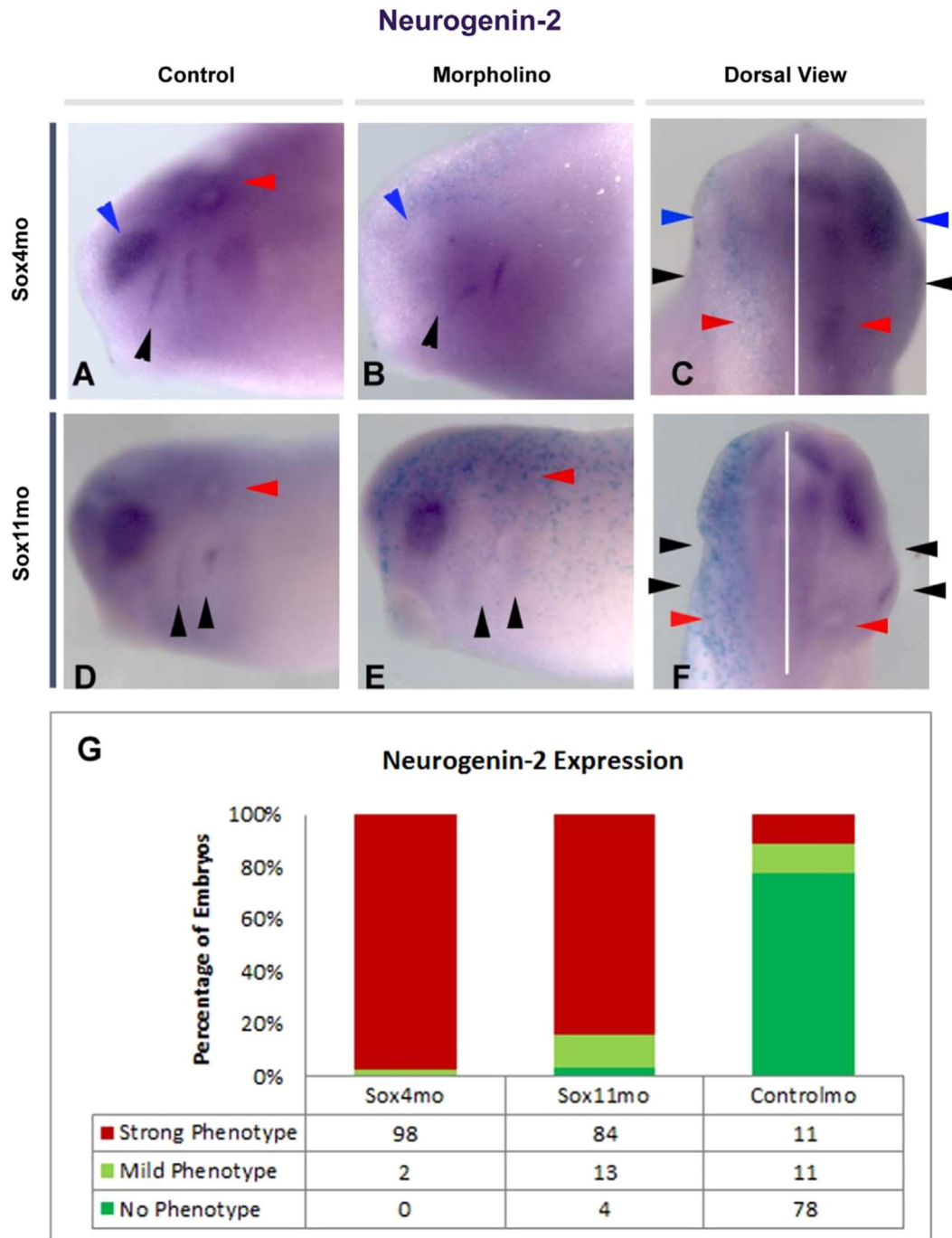
**Fig. 5. *Xenopus laevis* Sox4 and Sox11 regulate neural crest specifier genes FoxD3, Sox10, and Twist.** Functional knockdowns of Sox4 and Sox11 lead to the loss of neural crest specifiers on the injected side (shown by lacZ staining) of FoxD3 (A and B), Sox10 (C and D), and Twist (E and F). (I) Quantification of effects on FoxD3 expression: Sox4mo (n=36), Sox10mo (n=33), and Controlmo (n=23)  $\chi^2=51.8$  with 3 degrees of freedom. (J) Quantification of effects on Sox10 expression: Sox4mo (n=43), Sox11mo (n=20), and Controlmo (n=22)  $\chi^2=50.4$  with 4 degrees of freedom. (K) Quantification of effects on Twist expression: Sox4mo (n=73), Sox11mo (n=47), and Controlmo (n=24)  $\chi^2=90.2$  with 4 degrees of freedom. (G–H) Sox4mo injected embryos rescued with Sox4 mRNA by FoxD3 expression. (L) Quantification of effects with FoxD3: Sox4mo (n=67) and Sox11mo (n=30) ( $\chi^2=3.28$ ) with 2 degrees of freedom.



Neurogenin



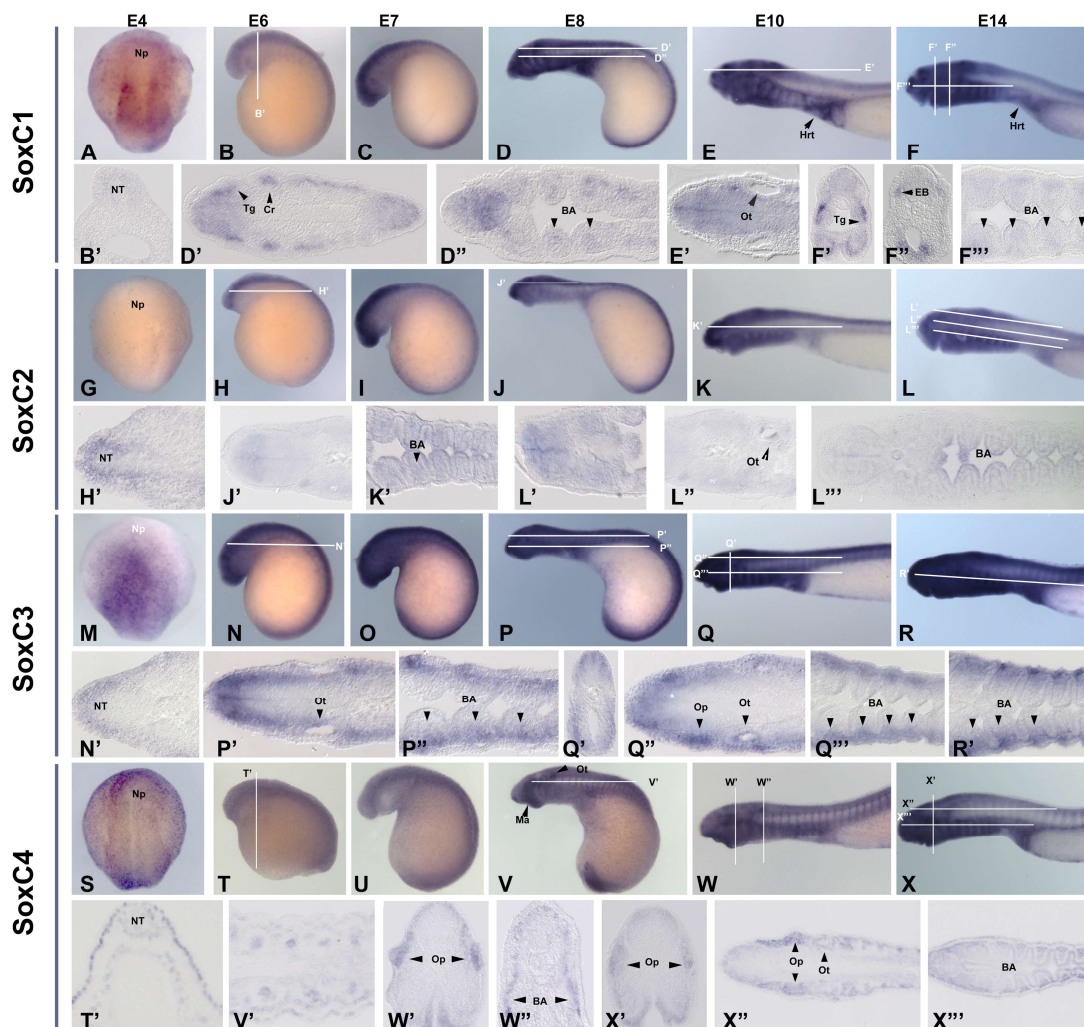
**Fig. 6. Loss of SoxC genes in lamprey causes defects in the cranial ganglia and branchial arches:** Knockdown of SoxC1 results in defects in neurogenin expression (A–F). At E8.5, (Dorsal: A) loss of expression in cranial ganglia (red arrow) and in the forming branchial arches (black arrow) on the injected side (B) loss of rostral cranial ganglia (red arrow), branchial arch expression (black arrow), and forming epibranchial ganglia. (C) On the control side, both cranial ganglia and branchial arches appear normal. At E10 (D–F), (Dorsal: D) defects in expression of dorsal root ganglia (black arrows) on the injected side, (E) absence of neurogenin expression in branchial arches (black arrows), (F) in contrast to their presence on the control side (black arrows). Knockdown of SoxC3 also results in defects in neurogenin expression (G–L). At E8.5, (Dorsal: G) loss of cranial ganglia expression (red arrow) on the injected side, (H) loss of cranial ganglia expression (red arrow) and no expression in forming branchial arches/epibranchial region (black arrow), (I) compared with the presence of both cranial ganglia (red arrow) and branchial arches/epibranchial ganglia (black arrow) on the uninjected side. At E9 [(Dorsal: J) and K] loss of expression in the acoustic ganglia (red), (K) loss of epibranchial ganglia, expression in both acoustic ganglia (red arrow), and branchial arches (black arrows). Knockdown of SoxC4 shows defects in neurogenin expression (M–R). At E8, (M, dorsal) loss of cranial ganglia expression (red) and acoustic ganglia/branchial arch stream expression (black arrow). At E10, [(Ventral: P) and Q] loss of branchial arch and epibranchial ganglia expression on the injected side (black arrows), (R) compared to retained expression in the branchial arches on the uninjected side. (S) Neurogenin: SoxC1mo(n=104), SoxC3mo(n=127), SoxC4mo(n=107), and Control mo(n=28) ( $\chi^2=67.7$  with 6 degrees of freedom).



**Fig. 7. *Xenopus laevis* Sox4 and Sox11 knock-downs affect Neurogenin expression later in development.** At stage 26, Sox4mo causes a loss of Neurogenin-2 expression in the branchial arches, otic vesicle, and eye (A–C). Sox11mo results in the loss of Neurogenin-2 expression in the branchial arches and otic vesicle (D–F). (H) Ngn1: Sox4mo (n=34), Sox11mo (n=52), and Controlmo (n=16).

**Sup. 1. Phylogenetic analysis of the SoxC Family.** Neighbor-joining analysis utilizing the HMG box protein sequence. Abbreviations *Bb*, *Branchiostoma belcheri*; *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Mm*, *Mus musculus*; *Pm*, *Petromyzon marinus*; *Xl*, *Xenopus laevis*;





**Sup. 2. SoxC1, C3, and C4 are expressed in premigratory neural crest and crest derivatives:** SoxC1, at 4 -14 days (A -F), is expressed as early as E4 (Dorsal view:A) on the neural folds. It continues to be expressed in the neural tube and developing pharyngeal arches beginning at E6 (B-F). At E8 (D), it is expressed in cranial ganglia (D') and in the mesenchyme/neural crest-derived portions of forming branchial arches (D''). At E10 and E14 (E & F), expression is very strong in the mesenchymal portions of the branchial arches, trigeminal ganglia, and heart. SoxC2 at 4 -14 days (G-L) is expressed throughout the neural tube from E5-E7 (G - I). From E8-E14(J-L), SoxC2 is also expressed in the endoderm-derived portions of the branchial arches. SoxC3 at 4 -14 days (M-R) is expressed in the posterior neural plate at E4 and in the neural folds, neural tube, premigratory and migratory neural crest, and ectoderm at E6 and E7 [(Dorsal view: M), N, O]. From E8 (P), expression persists in the ectoderm and neural tube and begins in the mesenchymal/neural crest derived portions of the branchial arches as well as in the otic vesicle (P', P''). At E10 (Q), expression persists in the branchial arches as well as the otic vesicle and cranial ganglia (Q'-Q'''). At E14 (R), SoxC3 is expressed in the branchial arch cartilage and the mesenchymal/neural crest-derived portion of the branchial arches. SoxC4 at 4 -14 days (S-X), is expressed early in the neural plate and ectoderm (S). Later, expression is seen in the neural folds and neural tube and persists in the ectoderm and endoderm at E6-E7(T, T' & U). At E8 (V), SoxC4 is highly expressed in the mandibular arch, optic vesicle, and somites. At E10 and E14 (W, X), SoxC4 is expressed in the mesodermal/neural crest derived

portions of the branchial arches. Np: Neural plate; O: Otic vesicle; Op: Optic vesicle; BA: Branchial Arch; Tg: Trigeminal Ganglia; Cr: Cranial Ganglia; Eb: Epibranchial ganglia; Ma: Mandibular Arch; Hrt: Heart.

### **Sup. 3. Full Length SoxC proteins**

#### SoxC1:

MVQQAGGRSIMDRPEHAAASRSDSEAEGSDPGSVAPGPLVGVLTESGAPGPGWCKTAS  
GHIKRPMNAFMVWSQIERRKIMEQSPDMHNAEISKRLGRRWKQLKESEKVPFIREAERLR  
LKHMA DYPDYKYRPRKKAKLAQQQQQQGSPKGTAGRSSSSSKSSGKVKAQQQQPKMG  
SSLLKEAAGGRVVRVAKRSPKGAAGERGKASASASSASSHAAAGAAVPSSPTLSIPGEAVD  
RLSFYEECGPGRER

#### SoxC3:

MVLHASGLPSSADDCLSADEMSDSEGS DLEPGSCMPSPAALSARVADTAGAVGSALGGG  
GGAGASSSGSGNGGQPDWCKTANGHIKRPMNAFMVWSQIERRKIMMESP DVHNAEISKR  
LGRRWKQLKDCEKVPFMREAERLRLKHMA DYPDYKYRPRKKAKTPGGGGGGGAAGVC  
AAAGSGPAS PQEKPPKSSKKGDSAKRSGGVSGGKSKAQKLPPHLRPPSGGRGLKTVSTKT  
VRDLLAPPQARHHHHHHHQLGNHHQHSSSQPTSPAAVPPSPTLSSGSAEGRESASFYEDIS  
GVRVTTIKIEPGSVSPSVPSVSTPMATMLSSGGAGGAAGGMDDSI LDFALSASERGELQT  
LGSIGLSTLDRDIDSLSDGSSASHFDFPDYCTPEVSELISGDWFESSITNMVFTY

#### SoxC4:

MRRPSQA ACTAATTTATGSIAPPPPPRLTGHIKRPMNAFMVWAQIERRRLMELSPEMHNA  
EISRRLGRLWRL LGDAEKKPFVREAERLRAQHMA DHPDYKYRPRKRVKAQPPATQLPPSS  
SSQRLPPHQPRHSGPPPPSSAARAKAAAAAAAAGGGARKRAVAHGRLPDDARRASDST  
GSSRSRD TTRS RPQRPASGGGSLCEQAARHDGARMEEDPRGDEGDGAALMSGRSRGYA  
GSNWYELDKEKVMEEEEKEEDEDEEMGRDVQLLGS LDRDVDSLSEGSSASHFDFPDLRS  
PEMNEILSGSPWLESSFSSLVFTY

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*Chapter 5***CONCLUSION**

The neural crest is an important cell type that is essential for the development and evolution of vertebrates. In this thesis, I evaluated neural crest migration and derivatives in lamprey along the anterior-posterior axis. Although present in lamprey, the cranial and trunk neural crest subpopulations are missing major derivatives such as the opposable jaw and the sympathetic chain ganglia in conjunction with the absence of the vagal and cardiac neural crest contributions. We found evidence of a trunk neural crest derived portions of the ENS in the apparent absence of vagal neural crest. The lamprey cranial NC GRN showed differences in the transcription factors that are important in cartilage formation in chicken. The transcriptome data showed that many genes normally enriched in the chicken cranial region were not similarly enhanced in lamprey and no lamprey specific cranial neural crest genes. These differences in the GRN and gene expression may account for variation in skull structure and the craniofacial skeleton of gnathostomes versus and agnathans. Although there are many differences in gene expression between jawed and jawless vertebrates, expression and function of many gene families are conserved. Amongst them are the SoxC subfamily whose early expression and role on neural crest specification is conserved. In this study, I propose that the lamprey represents an intermediate perspective in the stepwise development and evolution of the neural crest subpopulations by examining neural crest regionalization and gene circuits.

### ***Differences Between Cranial and Trunk Neural Crest Are Based on Variation in the NC-GRN***

The gnathostome neural crest is subdivided into several regions in gnathostomes (cranial, cardiac, vagal, trunk, and sacral neural crest). Based on our DiI labeling experiments, I found

only cranial and trunk neural crest populations. Cranial neural crest migratory pathways are similar to those observed in jawed vertebrates despite the lack of jaws. Cranial neural crest contributed to portions of the trigeminal ganglia and branchial arch derivatives. Interestingly, my results show that DiI injections must be done prior to E6.5 in order to label the cranial neural crest's contribution to the trigeminal ganglia. This is likely due to the fact that at E6.5, cranial neural crest cells have already begun delaminating and migrating. Trunk neural crest were observed in the fin mesenchyme, DRGs, spinal nerves, and in the typhlosole. To achieve more extensive DiI labeling of trunk neural crest, we performed DiI luminal tube fills at E8-8.5. Though the cranial neural crest populations have already migrated at this time, we excluded their contributions by only labeling more posterior regions so as not to label any structures anterior to the hindbrain.

The major difference between the cranial and trunk neural crest cells in all vertebrates is the capacity to form cartilage. In contrast to most gnathostomes, the lamprey skull is composed of a brain case and cartilage, but is not ossified. Some of the cranial and trunk neural crest derivatives in lamprey are conserved. Key orthologous transcription factors of the chicken cranial NC-GRN (*Tfap2c*, *SoxE*, and *Id*) are expressed in premigratory neural crest. *SoxEs* are essential for the invention of the neural crest and serve as key players in neural crest specification and migration. It is not surprising that this important element of the neural crest GRN—activation of *SoxEs* via *Tfap2*—is present in lamprey and important for cartilage formation. These transcription factors appear to be the basic for cranial neural crest cartilage formation which was expounded upon in jawed vertebrate cranial neural crest GRN. It is less likely that ancient ancestors to jawed and jawless vertebrates possessed the complete GRN and lamprey lost some of these elements. With the exception of *LZTS1*, other



candidate orthologs are present in the lamprey genome but not expressed in the neural crest. Some of these genes (Twist, Ets1, LHX5, and Dmbx1b) are co-expressed at later stages of neural crest formation in lamprey branchial arches, consistent with the possibility that the elaboration of the cranial GRN might have taken place through cooption of parts of differentiation programs to earlier portions of the network. Through our transcriptome analysis, we examined orthologs of candidate cranial genes in chicken. We found only 7 genes that were enriched in both species (Col9a1b, Naca, Tfp2b, eef1a2, Acp1, abtb2b, and Vill). Examining the expression patterns of members of the 20 genes that were most enriched in our cranial neural crest dataset, we found gene expression restricted to the cranial regions of the CNS, but absent in the neural crest. From this, we were unable to identify cranial neural crest genes that were unique to lamprey cranial neural crest. This further supports the hypothesis that agnathans possess a simpler cranial neural crest gene regulatory network, which may be important for the evolution of the neural crest subpopulations and structural changes inclusive of the skull and a proper jaw.

### ***Origins of the Cranial and Trunk Neural Crest***

Both the cranial and trunk neural crest populations are present in basal vertebrates, suggesting that they emerged in a common ancestor to jawed and jawless vertebrates. Yet it remains unclear whether the cranial or trunk subpopulation came first. Some speculate that the cranial neural crest is the most basal and the trunk neural crest lost the ability to form cartilage. Evidence in support of this comes from ostracoderms (a collective group of extinct and bony-armored agnathan) which had an exoskeleton armor that may have been derived from trunk neural crest. Furthermore, mesoderm as well as cultured chicken trunk neural

crest cells can be induced to differentiate into cartilage in vitro (Donoghue, Graham et al. 2008, Coelho-Aguiar, Le Douarin et al. 2013). Conversely, others posit that trunk neural crest cells were the rudimentary neural crest population and later added GRN elements which allowed for the cranial neural crest region to gain the ability to form cartilage (Mongera and Nusslein-Volhard 2013, Shimada, Kawanishi et al. 2013, Green, Simoes-Costa et al. 2015). This is supported by neural plate border cells in *Ciona* that undergo EMT and form pigment cells or bipolar tail neurons (Abitua, Wagner et al. 2012, Stolfi, Ryan et al. 2015). In amphioxus, cartilage is formed by mesendoderm expression of SoxE and exposure to FGFs (Jandzik, Garnett et al. 2015). Unlike vertebrates, SoxEs are not regulated by Tfap2. This role in cartilage formation was likely coopted by the cranial neural crest first by the expansion of the SoxE cis-regulatory elements and then by subsequent exposure to FGFs. Collectively, it is likely that the basic elements observed in the lamprey cranial cartilage NC GRN (SoxEs, Tfap2, Id) coopted the earlier mesendodermal role while the transcription factors expressed exclusively to gnathostomes (LMO4a, CSRNP, Brn3, Lhx5, Dmbx1, Ets1, Twist, and LZTS1) were likely further gained, refined, or moved earlier in the network. The relative scarcity of cranial factors in the lamprey cranial neural crest might suggest that the GRN underlying this cell population has undergone extensive elaboration from a regulatory standpoint. Thus, we propose that regionalization of the neural crest, with both the emergence of the new subpopulations and the expansion of the cranial GRN, played a crucial part in vertebrate evolution, culminating in the rise of the gnathostomes.

***Initial role of SCPs in ENS development followed by the cooption of Vagal NC***

The vagal neural crest may be a transitional hybrid population between the trunk and cranial neural crest not only in its AP positioning but also based on its cell-autonomy and plasticity (Kuo and Erickson 2011). The anterior vagal/cardiac neural crest (somites 1-3) is thought to be more cranial neural crest-like, while the posterior vagal neural crest is more trunk neural crest-like (somites 4-7). From our data, it is likely that the vagal NC emerged after the separation of jawed and jawless vertebrates. Lamprey cranial neural crest cells have the ability to contribute to the pharyngeal arches but we see no evidence for neural crest cells migrating to the outflow tract and ventricular septum.

Our data show that lamprey trunk neural crest cells have the ability to contribute to the enteric nervous system by making SCP. DiI labeling of the neural tube shows DiI along spinal nerves and contribute to 5-HT and acetylated tubulin positive cells in the typhlosole. Despite the absence of myelin in lamprey and certain key myelin genes (S100, Dhh), these spinal nerves are within the expression domain of some genes essential in Schwann cell maturation (Pmp22a, Emp2). Upon ablation of the dorsal portion of the neural tube and neural crest cells, 5-HT positive cells in the gut are decreased. The decrease in these 5-HT enteric cells, supports the trunk contributions to the ENS. Several scenarios may explain invention of the vagal neural crest. Lamprey “cranial” neural crest may be separated into two subpopulations based on their derivatives: the anterior region, which contributes to the trigeminal ganglia, and the posterior region, which enter the branchial arches. The latter resembles the vagal neural crest in the ability to enter the last branchial arches, but lacks the ability to migrate beyond the last branchial arch. It is possible that modifications to the cell response to signals allow for further migration. This population then gained the ability to

contribute to both the cardiac and enteric fates. To investigate the development of the vagal neural crest, comparative and functional analysis of signals that play a role in AP patterning (such as Hox genes) and their interactions could elucidate some information regarding development. Regulation of key transcription factors (such as Ret), and transcriptomic profiling of the vagal neural crest in gnathostomes could provide additional insights into the invention of the vagal NC. For example, Hox elements in lamprey are conserved with respect to the regulation of their expression along the neural axis. In particular, Hox groups 3-5 (Hoxb3 and Hoxb5) are known to be expressed in vagal regions of vertebrates. Hoxb3 has been shown to work as a tissue specific promoter for vagal neural crest and has conserved early expression in lamprey beginning at the third branchial arch at r5. Various factors such as those of the retinoic acids and hedgehog signaling pathways have been shown to interact and regulate specific vagal genes in gnathostomes such as Ret, which enable vagal neural crest cells to migrate (in particular when it interacts with GDNF from gut mesenchyme). Given the early conserved temporal expression of the Hox genes, it is possible that some but not all of the necessary components of the enteric gene battery are available and/or responsive. For example, Ret, Hand, and Ascl1(mash1) expression are not expressed in presumptive migrating vagal neural crest in lamprey, but are important in vertebrate ENS formation. However, both Phox2b and Ret are expressed in lamprey along the typhosole at different developmental time points. Given the lack of DiI labeled cells in the presumptive vagal region, absence of a cardiac septum, and the lack of Ret expression in those particular regions, it is most likely that the enteric programming of Phox2b and Ret are both still present and important in enteric development and are derived from the SCP but not present in migrating neural crest cells. In lamprey, many of these genes that are expressed in different

locations and may not be regulated in similar ways. In addition, lamprey may be missing certain important factors (such as desert hedgehog).

The absence of the vagal neural crest in lamprey, but the presence of NC derived ENS from the trunk neural crest suggests that the trunk neural crest may originally have been the source of ENS neurons. In gnathostomes, this function was later coopted by the vagal neural crest. Since other non-vertebrate chordates and protochordates also have enteric neurons, there must be a non-neural crest origin for the ENS. Thus, we cannot fully quantify the contributions to the ENS from the trunk neural crest in comparison to alternative sources. Unfortunately, ENS development has not been well-studied in non-vertebrate chordates or other deuterostomes, making it difficult to make maximum parsimonious comparisons. In insects, the ENS is believed to emerge from the neurogenic zones of the epithelium, to delaminate, migrate, and then proliferate. However, the closest organisms to the chordates in which the ENS has been closely examined are also deuterostomes, the echinoderms. *Holothuria glaberrima* (sea cucumber) are studied for their regenerative capability of their ENS. Their gastrointestinal ultrastructure is similar to vertebrates. However, their enteric innervation is quite different (as they possess four layers of gastrointestinal innervation within the gut wall). From luminal to adventitia they are the mucosal endocrine plexus, connective tissue plexus, basiepithelial plexus, and visceral plexus. The basiepithelial plexus appears to be conserved amongst all echinoderms and is in the same location as the submucosal plexuses in vertebrates (Garcia-Ararras, Rojas-Soto et al. 2001). This may be an example of convergent evolution giving rise to similar ultrastructure to vertebrates.

An interesting question is how or why the neural crest coopted the ability to form the ENS. As the new head hypothesis states, neural crest and placodes provided vertebrates with

the features and tools necessary to become more effective predators. It is tempting to speculate that the change in role from filter feeder to active predation provided an evolutionary advantage for more gut motility and modulation. As filter feeders, the rate of feeding is more constant such that an intricate enteric reflex may not be necessary. On the other hand, as predators, the organism may go through periods of feast and famine which require finer levels of gastrointestinal control and reflex. Food is no longer size restricted and, in the case of gnathostomes, no longer requires mechanical breakdown of food through mastication. Lamprey have both a neural crest and non-neural crest derived origins of the ENS which may support the filter feeder life style, as ammocetes, and parasitic life styles, post-metamorphosis, respectively. Similarly, sea cucumbers and other echinoderms are not pure filter feeders and may have evolved convergently to an ENS similar in organization to vertebrates. Thus, it is not surprising if the enteric ultrastructure is somewhat similar to vertebrates. There is even variation amongst the vertebrate lineages. Zebrafish, for example, only possess one set of ganglia plexuses (myenteric plexus) and do not have a submucosal layer. In vertebrates, the sympathetic chain ganglia, which is a collection of prevertebral neurons synapsing from different levels, are essential for sympathetic nervous system response inclusive of gastrointestinal regulation via the splanchnic nerves (i.e. Greater, Lesser, Least). During the “fight or flight” response, gastric motility is halted through constriction of smooth muscle. The lack of sympathetic chain ganglia in lamprey may shed light on the rationale as to why the combination of trunk neural crest and other ENS precursors was selected rather than a vagal and trunk neural crest combination. It is possible that without a sympathetic chain for relaying central sympathetic control to all levels, trunk neural crest (Schwann cell precursors) may provide segmental control of regions of the gastrointestinal

tract. With this segmental control, regionalization of Schwann cell precursors and preganglionic spinal nerve input in combination with other non-neural crest sources for basal enteric activity could be more advantageous than a sympathetic chain for central control and vagal neural crest for reflex control as a larval filter feeder. Conversely, it is possible that vagal neural crest, trunk neural crest, and non-neural crest derived ENS may have no evolutionary advantage and the evolution of the neural crest and vagal neural crest to the ENS was purely due to developmental drift. To investigate this hypothesis, examining the ultrastructure of non-vertebrate chordates such as *Ciona* and *Amphioxus* will be critical. Examining the regionalization of plexuses would be the first step to see if the distribution is optimal for the role of filter feeders or if it is only necessary for organisms with a predator-like role. Second, it would be helpful to assess neurotransmitter function and hormonal regulation of ENS function and activity since both lamprey and hagfish have a CCK-like and secretin-like system (Ostberg, Van Noorden et al. 1976).

***Effects of SoxCs on early neural crest specification may be due to effects on Wnt signaling***

Given the absence of the sympathetic chain ganglia and the different manner for generating the ENS than other vertebrates, it is not surprising that lamprey innervation and organization is unique. However, SoxC function is conserved in early neural crest specification and possibly in later neurogenesis. SoxCs (SoxC4 in particular) are also generally expressed in a region in which a presumptive sympathetic chain would form. Neurogenin2 has been said to initiate basic helix loop helix genes (such as *Mash1* and *Hand*) which in turn upregulate *Sox11* and *Sox4*, but we see that loss of SoxC genes results in the downregulation of Neurogenin2. From our results in decreasing neural crest specifiers, this

effect can be secondary. Ectopic expression of SoxCs in *Xenopus* and lamprey result in over expression of Neurogenin2, indicating that the SoxCs are capable of regulating neurogenesis. However, lamprey *Ascl1* (*mash1*) and *Hand* are not expressed in regions associated temporally or spatially with SoxCs, neurogenins, or sympathetic chain. This further bolsters the support for the absence of a sympathetic chain ganglia and the hypothesis that gnathostomes later recruited these functions to form sympathetic chain ganglia, but the main elements of neurogenesis are still present and similarly regulated.

In other vertebrates, Sox4 and Sox11 have complex interactions, having the ability to upregulate or downregulate Wnt signaling depending on partner coregulation. For example, Sox11 interacts with both NLK (negative regulator of WNT) and SMAD3 (positive stabilizer of  $\beta$ -catenin) in lymphoma cells. In cancer cells, the SoxCs have an opposing function of decreasing Wnt signaling for positive prognostic factors; however, in mice, SoxCs stabilize  $\beta$ -catenin via blocking GSK3 activity. By impacting Wnt signaling, we interrupted the neural crest GRN cascade resulting in an indirect effect on neural crest specifier genes. *Xenopus* knockdowns of Sox4 or Sox11 result in a minor expansion of the neural plate through Sox2 and Sox3 expression as well as down-regulation of neural crest border specifier genes Pax7 and Msx. Knockdown of these genes may have resulted in the decrease of Wnt signaling, decreasing neural plate border and thus neural crest specifier genes. Alternatively, loss of SoxCs allows for SoxB function to predominate the region, resulting in an inability for the cells to be specified and thus migrate. Furthermore, at later stages of development, little additional cell death is seen on the injected side compared to the control side with TUNEL assays. Regardless of the mechanism, there is a decrease in neural crest migration. This may explain the phenotypes in mice which died from knockouts of the



SoxC genes from cardiac defects and thymic aplasia. This is very similar to patients diagnosed with DiGeorge Syndrome (22q11 deletion) which is the failure of the development of the third and fourth pharyngeal pouch responsible for the parathyroid and thymus formation in addition to cardiac (i.e. truncus arteriosus, transposition of great vessels, ventricular septal defects, tetralogy of fallot) and facial abnormalities (cleft lip and palate). This raises the possibility that SoxCs are impacting the regulation of cardiac/vagal neural crest pathway. In addition to early expression in the neural folds, SoxCs are expressed throughout all the neural crest derived portions of the branchial arches inclusive of arches 3 and 4. Further investigation of genes involved in DiGeorge Syndrome such as TBX1 and the interaction with SoxC could elucidate some key effects on the vagal neural crest. Since we cannot observe a vagal/cardiac neural crest population, the absence of a cardiac phenotype was not noted in lamprey, while slight cardiac defects were noted in *Xenopus* knockdowns which were allowed to develop beyond initial collection point. Despite this discrepancy, the early expression and function upon neural crest specification is conserved amongst vertebrates. Further overexpression of SoxCs in lamprey result in neural tube defects, possibly due to the increase and stabilization of  $\beta$ -catenin resulting in an upregulation of E-Cad, which is a known cofactor of the SoxCs, and thus downregulation of N-cad resulting in open neural tube which could lead to spina bifida.

***Future work: Lamprey neural crest specific enhancers***

Although DiI is an effective method of tracing cell lineages, development of a neural crest tissue specific enhancer has been a long time goal of our laboratory. Ideally, SoxE (Sox9/10) enhancer would allow us to see all neural crest derivatives including a larger

population of Schwann cell precursors. Not only can this help validate the absence of vagal neural crest, but also serve as a means of expressing genes that are missing the lamprey neural crest GRN. Identifying enhancers that are functionally conserved across species is non-trivial. Attempts have been made to clone upstream elements of lamprey Sox9 and Sox10 without success. However, our collaborator Dr. Hugo Parker has found that zebrafish Hoxa2 and Crestin enhancers appear to mediate expression in neural crest cells amongst other cell types. Rather than utilizing an enhancer, the idea has been proposed to utilize CRISPR in order to insert GFP into an exon of Sox10 to enable tracing of Sox10 expression *in vivo*.

Having a neural crest specific enhancer will open new avenues for investigating lamprey neural crest migration, expressing constructs in a tissue specific manner, and isolating cells for single cell transcriptome analysis to further decipher the neural crest GRN. Once an enhancer is isolated, I would propose to sort these cells via FACs for transcriptome analysis of premigratory and migratory neural crest. From this, I would confirm candidate genes and view interacting players involved in neural crest migration in comparison to other vertebrates via ChIP seq. After validating these findings, I would misexpress the genes that are absent in a neural crest specific manner (e.g. known members of the cranial NC GRN, such as BRN3). Overexpression of Twist and Ets1a in 2 cell injection had no impact on early neural crest specifiers at E6-E7.5. However, in a small batch of Twist over-expression embryos, enlargement and atypical morphology was observed.

In addition to evaluating the GRN and transcriptomics, I would also return to more classical developmental biology through grafting experiments. We have attempted to graft *Xenopus* into lamprey and vice versa with very little success. In a case (n=1), *Xenopus* tissue was successfully integrated into the palate of the lamprey. Injection of isolated cells may be

beneficial in the ability to successfully integrate rather than our previous attempts of utilizing explants. Grafting vagal or cardiac neural crest from *Xenopus* into lamprey to view if they are capable of migrating into the gut would be useful. Conversely, it would be interesting to test if lamprey neural crest cells can be placed in the *Xenopus* vagal stream and contribute to the vagal neural crest. If they are unable to do so, I would follow up with the over-expression of missing genes and regrafting, hopefully identifying essential components to vagal neural crest programming.

Understanding the evolution of the neural crest in lamprey and its transitions to acquire the ability to form new derivatives holds the promise of providing insight into the emergence of vertebrate features. This in turn will contribute to understanding the organization of neural crest subpopulations and regionalization along the body axis. Observing neural crest in its current most ancestral form allows us to identify and appreciate the preserved architecture of certain elements of the network as well as the changes which were necessary to adapt to vertebrate life.

**APPENDIX: A****A FATE-MAP FOR CRANIAL SENSORY GANGLIA IN THE SEA LAMPREY**

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## Abstract

Cranial neurogenic placodes and the neural crest make essential contributions to key adult characteristics of all vertebrates, including the paired peripheral sense organs and craniofacial skeleton. Neurogenic placode development has been extensively characterized in representative jawed vertebrates (gnathostomes) but not in jawless fishes (agnathans). Here, we use *in vivo* lineage tracing with DiI, together with neuronal differentiation markers, to establish the first detailed fate-map for placode-derived sensory neurons in a jawless fish, the sea lamprey *Petromyzon marinus*, and to confirm that neural crest cells in the lamprey contribute to the cranial sensory ganglia. We also show that a pan-Pax3/7 antibody labels ophthalmic trigeminal (opV, profundal) placode-derived but not maxillomandibular trigeminal (mmV) placode-derived neurons, mirroring the expression of gnathostome Pax3 and suggesting that Pax3 (and its single Pax3/7 lamprey ortholog) is a pan-vertebrate marker for opV placode-derived neurons. Unexpectedly, however, our data reveal that mmV neuron precursors are located in two separate domains at neurula stages, with opV neuron precursors sandwiched between them. The different branches of the mmV nerve are not comparable between lampreys and gnathostomes, and spatial segregation of mmV neuron precursor territories may be a derived feature of lampreys. Nevertheless, maxillary and mandibular neurons are spatially segregated within gnathostome mmV ganglia, suggesting that a more detailed investigation of gnathostome mmV placode development would be worthwhile. Overall, however, our results highlight the conservation of cranial peripheral sensory nervous system development across vertebrates, yielding insight into ancestral vertebrate traits.

## INTRODUCTION

The neural crest (reviewed in Hall and Gillis, 2013; Prasad et al., 2012) and cranial neurogenic placodes (reviewed in Schlosser, 2006; Schlosser, 2010; Grocott et al., 2012; Graham and Shimeld, 2013) are transient, distinct embryonic cell populations whose derivatives include many key vertebrate characters, including the craniofacial skeleton and the paired peripheral sense organs. The conservation of the neural crest gene regulatory network across all vertebrates has been demonstrated by gene expression and morpholino-mediated functional knockdown studies in lampreys (Sauka-Spengler et al., 2007; Nikitina et al., 2008; Sauka-Spengler and Bronner-Fraser, 2008), which, together with hagfishes, are the only surviving representatives of the jawless fishes (agnathans). Lampreys and hagfishes—the cyclostomes (reviewed in Osório and Rétaux, 2008; Shimeld and Donoghue, 2012)—occupy a key phylogenetic position for understanding vertebrate development and evolution, since any traits shared by jawed vertebrates (gnathostomes) and one or both cyclostome lineages can parsimoniously be assumed to have been present in the vertebrate ancestor. Although cranial neurogenic placode development has been extensively studied in representative gnathostomes (reviewed in Schlosser, 2006; Schlosser, 2010; Grocott et al., 2012; Graham and Shimeld, 2013), relatively little information is available on lampreys (e.g. von Kupffer, 1895; Damas, 1944; Fisk, 1954; Murakami et al., 2001; Neidert et al., 2001; McCauley and Bronner-Fraser, 2002).

In gnathostomes, all cranial neurogenic placodes originate from a specialized region of ectoderm around the anterior neural plate called the “pan-placodal primordium” or “pre-placodal region” (reviewed in Streit, 2007; Ladher et al., 2010; Schlosser, 2010; Grocott et al., 2012). Within the developing pre-placodal region, the rostral-caudal expression of

mutually repressive members of the Pax family of paired domain transcription factors seems to be essential for regional identity and subsequent development of individual placodes: Pax6 for the “anterior” placodes (adenohypophyseal, olfactory, lens); Pax2/5/8 for the “posterior” placodes (otic, lateral line, epibranchial); and Pax3 for the “intermediate” placodes (trigeminal) (reviewed in Schlosser, 2010; Grocott et al., 2012). Similar *Pax* family gene expression patterns have been reported for lamprey placodes (Murakami et al., 2001; McCauley and Bronner-Fraser, 2002; Osório et al., 2005). However, the existence of two molecularly distinct trigeminal placodes is sometimes overlooked: in birds and mammals, Pax3 is expressed by and required for the differentiation of the ophthalmic trigeminal (opV) placode and opV placode-derived neurons in the ophthalmic lobe of the trigeminal ganglion (Stark et al., 1997; Baker et al., 1999; Xu et al., 2008; Dude et al., 2009), while the Pax3-negative maxillomandibular trigeminal (mmV) placode gives rise to Pax3-negative neurons in the maxillomandibular lobe of the same ganglion (D'Amico-Martel, 1982; D'Amico-Martel and Noden, 1983; Xu et al., 2008).

In lampreys, as in gnathostomes, the ophthalmic trigeminal (opV, V1) nerve transmits somatosensory information from the rostral part of the head, while the maxillomandibular trigeminal (mmV, V2/3) nerve performs the same function for the upper and lower lips and velum (see Kuratani et al., 1997; Kuratani et al., 2004; Murakami and Watanabe, 2009; Oisi et al., 2013). In anamniotes, separate ‘profundal’ and ‘trigeminal’ ganglia (fused in some groups) have been described, but *Pax3* expression in the profundal placode in representatives of the three major gnathostome lineages (cartilaginous fishes, and lobe-finned and ray-finned bony fishes) confirms the previously proposed hypothesis that the anamniote profundal placode and ganglion are homologous, respectively, with the

amniote opV placode and the ophthalmic lobe of the amniote trigeminal ganglion (O'Neill et al., 2007; Schlosser and Ahrens, 2004; Modrell et al., 2011). OpV and mmV placodes have been described in lampreys (von Kupffer, 1895; Damas, 1944; Fisk, 1954), but it remains unclear whether these placodes (or the neurons derived from them) can be distinguished via Pax3 expression, as would be expected given the assumed homology of cyclostome and gnathostome opV/profundal ganglia (Northcutt, 1979; Koyama et al., 1987; Wicht and Northcutt, 1995; Kuratani et al., 1997; Kuratani et al., 2004; Murakami and Watanabe, 2009). To date, a single *Pax3/7* subfamily gene has been isolated from three lamprey species: an apparent *Pax7* ortholog in the sea lamprey *Petromyzon marinus* (McCauley and Bronner-Fraser, 2002) (also see O'Neill et al., 2007), and an unresolvable *Pax3/7* gene in both the river lamprey *Lampetra fluviatilis* (Osório et al., 2005) and the Arctic lamprey *Lethenteron camtschaticum* (junior synonym *Lethenteron japonicum*) (Kusakabe et al., 2011). Similarly, a single *Pax3/7* gene was reported in the inshore hagfish *Eptatretus burgeri* (Ota et al., 2007). Although *Pax3/7* expression was described in the lamprey “trigeminal” placode and/or ganglion (McCauley and Bronner-Fraser, 2002; Osório et al., 2005), no distinction was made between opV and mmV placodes/ganglia.

Here, we have used neuronal differentiation markers and DiI labeling to construct the first detailed fate-map of neurogenic placodes in an agnathan, the sea lamprey *P. marinus*. In addition, after labeling premigratory neural crest cells up to a day earlier than in a previous study (McCauley and Bronner-Fraser, 2003), we show that cranial sensory ganglia in the sea lamprey also contain neural crest-derived cells. Our results suggest that the development of neurogenic placodes and cranial sensory ganglia is in general highly conserved across all vertebrates, including expression in the opV placode and opV placode-derived neurons of



the single Pax3/7 ortholog in lampreys and Pax3 in gnathostomes. Unexpectedly, however, our data suggest that upper lip-innervating and lower lip/velum-innervating mmV neurons, which are spatially segregated within the lamprey mmV ganglion (Koyama et al., 1987; Murakami and Kuratani, 2008), may originate from spatially segregated precursors, with opV neuron precursors sandwiched between the two. Although this may be a derived feature of lampreys, maxillary and mandibular trigeminal neurons are spatially segregated in the gnathostome mmV ganglion, suggesting that more detailed investigation of the mmV placode could reveal spatial segregation of maxillary and mandibular trigeminal neuron precursors in gnathostomes.

## **MATERIALS AND METHODS**

### **Embryo collection**

*P. marinus* eggs were collected from adults and fertilized as described (Nikitina et al., 2009). Embryos were maintained at 18°C in 0.1x or 1x Marc's modified Ringer's (MMR) solution.

### **Phylogenetic analyses**

To analyze the orthologous/paralogous relationships of the Pax3/7 family of transcription factors in chordates, phylogenetic analyses were performed under Bayesian and coalescence-based frameworks using amino acid sequences available from GenBank (National Center for Biotechnology Information), Ensembl ([www.ensembl.org](http://www.ensembl.org)) or SkateBase

([www.skatebase.org](http://www.skatebase.org); Wang et al., 2012). Detailed methodologies and a table of species names and accession numbers are available in supplemental materials.

### **DiI labeling**

DiI labeling was performed as described (Nikitina et al., 2009), with some modifications. Briefly, embryonic day (E) 5-7 embryos (Piavis stages 11-12: late neurula) (Piavis, 1961; Richardson and Wright, 2003) were manually dechorionated in 0.1x MMR, and then immobilized and oriented in 1x MMR in 18-mm Petri dishes that were either agarose-coated with depressions or lined with a fine mesh. Embryos were pressure-injected using glass capillary tubes filled with 0.5 mg/ml of Cell Tracker-CM-DiI (Invitrogen) diluted in 0.3M sucrose (from a 5  $\mu$ g/ $\mu$ l stock diluted in ethanol). They were allowed to recover in 1x MMR for 24 hours, and then individually transferred to an uncoated Petri dish containing 0.1x MMR and allowed to develop to E16-21 (Piavis stages 15-17: i.e., from embryos with a full complement of pharyngeal pouches, through to embryos with open gill slits and eyespots) (Piavis, 1961; Richardson and Wright, 2003). Embryos were periodically checked and imaged throughout, and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour at room temperature.

### **Generation of the fate-map**

Individual images (taken at the same magnification) were superimposed onto template embryos at E6-7 and E20-21. Using Adobe Illustrator, DiI-labeled regions were

outlined onto the template. Maps combining all labeled regions were generated for each placode and its associated ganglion, or for a combination of placodes and ganglia.

### **Immunohistochemistry**

Immunostaining was performed as described (Nikitina et al., 2009) with slight modifications; embryos were incubated overnight at 4°C in primary antibody in blocking solution (10% sheep serum in PBS with 0.1% Triton X-100); secondary antibodies were also incubated overnight at 4°C. Histochemical reactions were performed as described (Patel, 1994). Before imaging, embryos were cleared through a glycerol series into 70% glycerol in PBS. Primary antibodies: 1:50 HNK-1 (mouse IgM, clone 3H5, Developmental Studies Hybridoma Bank); 1:500 anti-HuC/D (mouse IgG2b; Invitrogen); 1:200 anti-neurofilament-M (mouse IgG2a; Invitrogen); 1:200 anti-Pax3/7 (clone DP312; Davis et al., 2005). (The Developmental Studies Hybridoma Bank was developed under the auspices of the NICHD and is maintained by the University of Iowa, Department of Biological Sciences, Iowa City.) Secondary antibodies: 1:1000 Alexa<sup>488</sup>-conjugated goat anti-mouse IgG and/or Alexa<sup>594</sup>-conjugated goat anti-mouse IgG (Invitrogen), or 1:600 horseradish peroxidase-conjugated or alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch).

### **Histology**

For cryosectioning, embryos were incubated in PBS with 5% sucrose for 4 hours at room temperature. After overnight incubation at 4°C in 15% sucrose in PBS, they were transferred into pre-warmed 7.5% gelatin in 15% sucrose in PBS and incubated for 1-4 hours

at 37°C, then oriented and embedded in molds, frozen by immersion in liquid nitrogen or a dry ice-isopentane solution for 30 seconds, and cryosectioned at 12-16  $\mu\text{m}$ . Gelatin was removed by a 5-minute incubation in PBS pre-warmed to 37°C. For paraffin wax sectioning, embryos were dehydrated into 100% methanol, cleared by step-wise transfer into HistoSol (National Diagnostics), embedded by step-wise transfer into Paraplast at 60°C (Fisher Scientific), and sectioned at 8-12  $\mu\text{m}$  using a rotary microtome. Slides were de-waxed in HistoSol and rehydrated into PBS through a graded ethanol series. After immunostaining, sections were counterstained with the nuclear marker DAPI (1 ng/ml) (Invitrogen) and mounted in Fluoromount G (Southern Biotech).

## RESULTS

### Development of cranial sensory ganglia in the sea lamprey

The developing cranial sensory ganglia in *P. marinus* embryos were visualized by whole-mount immunostaining for the neuronal Elav RNA-binding protein family members HuC/D (Hinman and Lou, 2008) (Fig. 1) and identified according to established descriptions of neurogenic placode and cranial ganglion development in the European brook lamprey *Lampetra planeri* (also referred to as *P. planeri*, *Ammocoetes planeri*) (von Kupffer, 1891; von Kupffer, 1895; Fisk, 1954), the river lamprey *Lampetra fluviatilis* (Damas, 1944) and the Arctic lamprey *Lethenteron camtschaticum* (*Lethenteron* is a subgenus of *Lampetra*; junior synonyms include *Lethenteron japonicum* and *Lampetra japonica*) (Kuratani et al., 1997; Murakami and Watanabe, 2009). Starting at embryonic day (E) 8 (Piavis stage 12/13;

Piavis, 1961; Richardson and Wright, 2003), HuC/D was observed in the neural tube and, more weakly, in presumptive opV and/or mmV placode-derived neurons (Fig. 1A,B). By E10 (Piavis stage 14), HuC/D expression revealed the separate opV and mmV ganglia; the small presumptive anterior lateral line (aLL) ganglion lying immediately dorsocaudal to the geniculate ganglion (i.e., the first epibranchial placode-derived ganglion, dorsal to the first pharyngeal pouch); and the very large posterior lateral line (pLL) ganglion lying immediately dorsocaudal to the petrosal ganglion (i.e., the second epibranchial placode-derived ganglion, dorsal to the second pharyngeal pouch) (Fig. 1C). Fig. 1D-H show the further development of the cranial sensory ganglia between E12-20 (Piavis stages 14-17), now including the developing chain of nodose ganglia (i.e., the third and more caudal epibranchial placode-derived ganglia, which form dorsal to the third and more caudal pharyngeal pouches), as well as dorsal root ganglia (Fig. 1F-H; compare with Figs. 7a and 8a from Kuratani et al., 1997). By E20 (Fig. 1H-J), almost all cranial sensory ganglia could be distinguished except the vestibuloacoustic ganglion (also unidentified in Kuratani et al., 1997), which lies medial to the otic vesicle. The whole-mount HuC/D immunostaining data at E20 are summarized in schematic form in Fig. 1I. HuC/D immunostaining on transverse serial sections (Fig. 1K) confirmed the presence of a large, seemingly contiguous ganglionic complex extending rostral and medial to the otic vesicle. This complex most likely comprises the fused geniculate and aLL ganglia, followed by the vestibuloacoustic ganglion medial to the otic vesicle and perhaps also the even more medial intracapsular ganglion (i.e., the second ganglion of the aLL nerve; Koyama et al., 1990), which in the adult lamprey is located within the otic capsule, immediately medial to the vestibuloacoustic ganglion (Koyama et al., 1990).

### **A pan-Pax3/7 antibody labels opV placode-derived neurons**

During neurogenic placode development in gnathostomes, only the opV placode and opV placode-derived neurons express Pax3 (Stark et al., 1997; Baker et al., 1999; Schlosser and Ahrens, 2004; O'Neill et al., 2007; Modrell et al., 2011), which is required for opV placode development and opV neuron differentiation (Dude et al., 2009). Detailed phylogenetic analyses of the Pax3/7 subfamily of transcription factors, which included Pax3/7 protein sequences from one hagfish (Ota et al., 2007) and three lamprey species (McCauley and Bronner-Fraser, 2002; Osório et al., 2005; Kusakabe et al., 2011), showed a well-supported cyclostome Pax3/7 clade and separate gnathostome Pax3 and Pax7 clades (Fig. 2). However, the relationships between the cyclostome Pax3/7 clade and the gnathostome Pax3 and Pax7 clades were unresolvable (Fig. 2).

We wished to determine whether lamprey Pax3/7, like gnathostome Pax3, is expressed by opV but not mmV placode-derived neurons. To address this question, we used a cross-reactive Pax3/7 antibody (Davis et al., 2005) to immunostain *P. marinus* embryos in whole-mount. This antibody has demonstrated broad species cross-reactivity to Pax3/7 proteins in arthropods, invertebrate chordates and vertebrates (Davis et al., 2005; Somorjai et al., 2012; Minchin and Hughes, 2008; Curran et al., 2010) and its core epitope, PD(V/I)YTREE (Davis et al., 2005), is present in the homeodomain of the *P. marinus* Pax3/7 protein (McCauley and Bronner-Fraser, 2002). At E5.5, Pax3/7-positive cells were observed primarily in dorsal regions of the developing brain (Fig. 3A). By E6.5, stronger Pax3/7 expression was found along the dorsal neural tube and also adjacent to it, presumably in migrating neural crest cells (Fig. 3B). Between E8 and E10, a discrete patch of Pax3/7-positive cells appeared in a pattern and location similar to the opV ganglion (Fig. 3C,D;

compare with Fig. 1A,B). By E12, this patch strongly expressed Pax3/7 (Fig. 3E). Double immunostaining for Pax3/7 and HuC/D in whole-mount (Fig. 3F), followed by coronal sectioning (Fig. 3G,H), confirmed that Pax3/7 expression was restricted to the developing opV ganglion and excluded from the mmV ganglion. Taken together with information on gnathostome Pax3 expression (Stark et al., 1997; Baker et al., 1999; Schlosser and Ahrens, 2004; O'Neill et al., 2007; Modrell et al., 2011) and function (Dude et al., 2009), these data support an evolutionarily conserved role for Pax3 in patterning the opV placode and ganglion in all vertebrates.

### **OpV neuron precursors are initially sandwiched between two separate domains of mmV neuron precursors**

The maxillary and mandibular branches of the mmV (V2/3) nerve are not comparable between lampreys and gnathostomes (see Kuratani et al., 1997; Shigetani et al., 2002; Kuratani et al., 2004; Murakami and Watanabe, 2009; Oisi et al., 2013), and hence we have followed here the nomenclature proposed by Oisi et al. (2013) (see Supplementary Figure 8 in Oisi et al., 2013), in which “V2/3A” designates the upper lip-innervating anterior branch and “V2/3B” the lower lip/velum-innervating posterior branch of the lamprey mmV nerve (see Kuratani et al., 1997; Murakami and Watanabe, 2009; Oisi et al., 2013). We used the vital lipophilic dye DiI to label discrete regions of cranial ectoderm at E6-7 (late neurula; Piavis stages 11-12), and followed subsequent development for 12-14 days, to approximately E18-21 (Piavis stage 17). In embryos in which DiI was injected into a broad patch of anterodorsal head ectoderm (n=26), represented by the dotted red line in Fig. 4A, DiI was observed in the condensing opV and mmV ganglia by 12 dpi (E18-19) (Fig. 4B; compare

with Fig. 1G,H). Furthermore, ophthalmic (V1) and upper lip-innervating (V2/3A) nerve branches, originating, respectively, from the opV and mmV ganglia, were also labeled with DiI (Fig. 4B arrows and inset). After sectioning in an oblique plane to include both ganglia, HuC/D immunostaining confirmed that the DiI-positive cells were located in the opV and mmV ganglia (Fig. 4C,D).

More focal DiI injections at E6-7 within this broader region allowed us to distinguish the location of opV and mmV neuron precursors. After DiI injection into a roughly central sub-region of the broader domain, outlined with the yellow dotted line in Fig. 4E (compare with the broader domain outlined in red in Fig. 4A), DiI-labeled cells were later observed only in the opV ganglion (Fig. 4F). To our surprise, mmV neuron precursors were found at E6-7 in two spatially segregated domains, correlating with the position of labeled neurons within the mmV ganglion at E20-21. A region of ectoderm anteroventral to (but partially overlapping with) the patch of opV neuron precursors gave rise to neurons in a relatively small, rostral domain of the mmV ganglion at E20-21, potentially corresponding to lower lip/velum-innervating V2/3B neurons (Kuratani et al., 2004; Murakami and Kuratani, 2008) (n=3; Fig. 4G,H; schematized in light green in Fig. 4K,L). In contrast, a region of ectoderm caudal to the patch of opV neuron precursors gave rise to neurons in a larger, caudal domain of the mmV ganglion at E20-21, potentially corresponding to upper lip-innervating V2/3A neurons (Kuratani et al., 2004; Murakami and Kuratani, 2008) (n=6; Fig. 4I,J; schematized in dark green in Fig. 4K,L).



### **Development of epibranchial and lateral line ganglia**

DiI labeling at E6-7 of cranial ectoderm caudal to the region containing opV and mmV precursors revealed the fate-map for the epibranchial and lateral line ganglia (summarized in Fig. 5A,F,J,O,T). In all embryos labeled in the area indicated in Fig. 5A (n=9), DiI was seen 14 days later (E20-21) in the geniculate/aLL ganglion complex rostral to the otic vesicle and also, in most cases (n=7/9), in the vestibuloacoustic/intracapsular ganglion complex, medial to the otic vesicle. An example of an embryo with DiI in both ganglionic complexes is shown in Fig. 5B-E (whole-mount images: Fig. 5B,C; HuC/D-immunostained transverse sections: Fig. 5D,E).

Ectoderm located caudal and ventral to geniculate/aLL-vestibuloacoustic precursors (outlined in Fig. 5F) contributed to the petrosal ganglion (n=3). An example is shown in Fig. 5G-I (whole-mount images: Fig. 5G,H; HuC/D-immunostained horizontal sections: Fig. 5I). Ectoderm located dorsal to petrosal precursors and caudal to geniculate/aLL-vestibuloacoustic precursors (outlined in Fig. 5J) contributed to the pLL ganglion (n=8). In an example of a focal injection within this area (Fig. 5K-N), DiI was also seen in the pLL ganglion and the pLL nerve, both in whole-mount (Fig. 5L; inset) and on HuC/D-immunostained transverse sections (Fig. 5M,N).

Ectoderm located caudal to petrosal and pLL precursors (Fig. 5O) contributed to the first four nodose ganglia. In the embryo shown in Fig. 5P (labeled at E7), DiI-positive cells were observed in both the third and fourth nodose ganglia (Fig. 5Q). Transverse sections immunostained with the carbohydrate epitope antibody HNK1, which is expressed in lamprey cranial sensory ganglia (Hirata et al., 1997; Horigome et al., 1999), confirmed the

localization of DiI-positive cells specifically within ganglia (Fig. 5R; inset). The expression of HNK1 (which does not label cranial neural crest cells in lamprey; Hirata et al., 1997; Horigome et al., 1999) largely overlapped with HuC/D immunostaining in cranial sensory ganglia (Fig. 5S).

Our DiI-labeling data also reveal regions of overlap between the precursors for different placodes at neurula stages (E6-7), shown in schematic form in Fig. 5T. Such overlap is also seen in fate-maps for gnathostome embryos at similar stages (see discussion in Pieper et al., 2011).

Taken together, these findings provide the first fate-map for placode-derived neurons in the cranial sensory ganglia of the embryonic lamprey.

### **Neural crest-derived cells are found in cranial sensory ganglia**

A previous *in vivo* DiI-labeling study in *P. marinus* performed at E6 concluded that neural crest cells do not contribute to the cranial sensory ganglia of lampreys (McCauley and Bronner-Fraser, 2003). This was surprising, given that neural crest cells in gnathostomes form the satellite glia of all cranial sensory ganglia, plus somatosensory neurons in opV and mmV ganglia and the root ganglia of several cranial nerves (e.g. Yntema, 1943; Yntema, 1944; Hamburger, 1961; Narayanan and Narayanan, 1980; Ayer-Le Lièvre and Le Douarin, 1982; D'Amico-Martel and Noden, 1983; Kious et al., 2002; Harlow et al., 2011; Quina et al., 2012). Since this was a negative result, we revisited this question by labeling neural crest precursors in the dorsal neural tube at earlier neurula stages (E5-6; Piavis stage 11).

DiI injection at E5 into the presumptive rostral hindbrain labeled neural crest cells that colonized the mmV ganglion (n=10) and peripheral nerves (presumptive Schwann cells;

Nakao and Ishizawa, 1987). In an example shown in Fig. 6A-F, DiI was injected into the dorsal neural tube at early E5 (Fig. 6A). By 5 dpi (E10), neural crest cells were observed in the optic, trigeminal and mandibular arch regions (Fig. 6B). By 11 dpi (E16), DiI-positive cells were seen in the region of the mmV ganglion and along presumptive nerves (Fig. 6C). Transverse sections at different axial levels, immunostained for neurofilament, showed DiI-labeled neural crest cells around the eye and scattered on the upper lip-innervating V2/3A nerve (Fig. 6D), as well as within the mmV ganglion and scattered along the lower lip/velum-innervating V2/3B nerve (Fig. 6E). DiI-positive cells were also observed in the cartilage of the branchial baskets, confirming successful neural crest labeling (Fig. 6F).

DiI injection at E5-6 into the dorsal neural tube in the vagal region revealed that neural crest cells colonize the nodose ganglia (n=7). In an example shown in Fig. 6G-L, at 1 dpi (E7), DiI-labeled cells were still largely restricted to the dorsal neural tube (Fig. 6G). However, by 3 dpi (E8-9), DiI-positive neural crest cells were observed ventral to the neural tube (Fig. 6H), while by 9 dpi (E14), they were found dorsal to the developing branchial arches (Fig. 6I). By 13 dpi (E18-19), HuC/D immunostaining on transverse sections revealed DiI-positive cells in nodose ganglia (Fig. 6J-L). Taken together, these results demonstrate that neural crest cells colonize cranial sensory ganglia (and give rise to presumptive glial cells) in agnathans.

## DISCUSSION

In recent years, the key transcription factors and signaling pathways involved in patterning the preplacodal region and neurogenic placodes have been elucidated in representative gnathostomes (chick, mouse, *Xenopus*, zebrafish; reviewed in Streit, 2007; Ladher et al., 2010; Schlosser, 2010; Grocott et al., 2012). Molecular developmental studies in lampreys help shed light on the developmental processes and mechanisms that are shared between agnathans and gnathostomes, hence likely to have been inherited from the vertebrate ancestor (see Osório and Rétaux, 2008; Shimeld and Donoghue, 2012). Several studies have described neurogenic placode, cranial sensory ganglion and nerve development in different lamprey species, based on histology (e.g. von Kupffer, 1891; von Kupffer, 1895; Damas, 1944; Fisk, 1954) or whole-mount axonal immunostaining (Kuratani et al., 1997; Kuratani et al., 1998; Barreiro-Iglesias et al., 2008). Here, we DiI-labeled different regions of late neurula-stage cranial ectoderm to provide the first detailed fate-map for placode-derived neurons in cranial sensory ganglia in the sea lamprey *P. marinus*. This was coupled with immunostaining for neuronal markers to describe the precise spatiotemporal development of cranial sensory ganglia.

Based on our findings, we define key stages of neurogenic placode development in *P. marinus*. Our fate-map suggests that at E6-7, the precursors for ophthalmic trigeminal (opV) neurons and both groups of maxillomandibular (mmV) neurons, i.e., upper lip-innervating V2/3A neurons and lower lip/velum-innervating V2/3B neurons (Oisi et al., 2013), are already largely separable (albeit with some overlap between opV and presumptive V2/3B neuronal precursors; see next section). In contrast, the more caudally located precursors for epibranchial, otic and lateral line placode-derived neurons still show extensive

overlap at this stage, suggesting that individual placode specification from a larger common placode field is ongoing (as seen in similar early-stage fate-maps in chick and *Xenopus*; Streit, 2002; Xu et al., 2008; Pieper et al., 2011). Therefore, we hypothesize that the pre-placodal region is being established during E4-5 (late gastrula-early neurula), followed by an extended period of segregation of individual placodes from a larger common field from E5-6 to E8-9. Immunostaining using a cross-reactive Pax3/7 antibody shows that the pan-vertebrate ophthalmic trigeminal (opV/profundal) marker Pax3 (lamprey Pax3/7: this study; gnathostome Pax3: Stark et al., 1997; Baker et al., 1999; Schlosser and Ahrens, 2004; O'Neill et al., 2007; Modrell et al., 2011) and the neuronal marker HuC/D first begin to be expressed in the developing ganglia from E8. Thus, there is rapid progression to neurogenesis upon placode formation, with ganglion formation well underway by E10, suggesting that the key stages for the regulation of placode-derived neuron differentiation are E7-10.

### **Spatial segregation of lamprey mmV neuronal precursors may prefigure later spatial segregation and somatotopy within the lamprey mmV ganglion**

The somatotopy of lamprey mmV nerve projections is reflected by the spatial segregation of different afferents within the mmV ganglion: lower lip/velum-innervating (V2/3B) neurons are found in the rostral part of the ganglion, while upper lip-innervating (V2/3A) neurons are located in the caudal part of the ganglion (Koyama et al., 1987; Kuratani et al., 2004; Murakami and Kuratani, 2008). Intriguingly, our fate-map suggests that their precursors are similarly spatially segregated in two discrete patches at late neurula stages, with V2/3B precursors located rostral to V2/3A precursors, and opV neuron precursors sandwiched between them. We have tentatively identified the anteroventral pool of mmV

neuron precursors (rostral to but partially overlapping with opV neuron precursors) as lower lip/velum-innervating V2/3B neuron precursors, because the neurons formed by this pool are confined to a small, rostral domain of the mmV ganglion, which seems to correspond to the location of lower lip/velum afferents as defined by dextran-biotin nerve-tracing in *Lethenteron camtschaticum* (*Lethenteron japonicum*) (Kuratani et al., 2004; Murakami and Kuratani, 2008). Similarly, we have tentatively identified the caudal patch of mmV neuron precursors at neurula stages (caudal to opV neuron precursors) as upper lip-innervating V2/3A precursors, because the neurons formed by this pool are confined to a larger, caudal domain of the mmV ganglion that seems to correspond to the location of upper lip afferents (Kuratani et al., 2004; Murakami and Kuratani, 2008). If this interpretation is correct, the somatotopy of the lamprey mmV ganglion may reflect a very early developmental distinction between neurons destined to innervate the upper lip versus the lower lip/velum, and perhaps even the induction of separate placodes for these neurons.

Previous histological studies in lamprey embryos have described morphologically identifiable opV and mmV placodes, with the opV placode located immediately rostrally to the mmV placode (von Kupffer, 1895; Damas, 1944; Fisk, 1954). Therefore, whether or not these two pools of mmV neuron precursors represent distinct V2/3A and V2/3B placode precursors, differential growth, and/or morphological movements associated with optic cup evagination, must bring these two patches of ectoderm together, caudal to the patch of opV placode precursors, to form the mmV placode identified morphologically in previous studies (von Kupffer, 1895; Damas, 1944; Fisk, 1954). In *Xenopus*, mmV placode precursors (which in this species express *Pax6*; Schlosser and Ahrens, 2004) initially lie rostral to opV placode precursors (Pieper et al., 2011), in contrast to the situation in chick (Xu et al., 2008). In

*Xenopus*, unlike in chick, both opV and mmV placodes develop relatively close to the optic cups; it has been proposed that morphological movements associated with eye evagination displace the lateral part of the rostral-most pre-placodal ectoderm ventrocaudally, such that after neural tube closure, the mmV placode is induced ventral and caudal to the opV placode (Pieper et al., 2011).

Although the spatial segregation of mmV neuron precursors may of course be a derived feature of lampreys, the mmV nerve in gnathostomes also exhibits somatotopy, with maxillary and mandibular neurons spatially segregated in the mmV ganglion. Nerve-tracing experiments have shown that maxillary and mandibular neurons are physically separated within the snake mmV ganglion by a septum of connective tissue and blood vessels (Molenaar, 1978). Maxillary and mandibular neurons are also spatially segregated within the maxillomandibular lobe of the trigeminal ganglion in birds, mammals and turtles, albeit with some overlap (Dubbeldam and Veenman, 1978; Noden, 1980a; Noden, 1980b; Erzurumlu and Jhaveri, 1992; Rhinn et al., 2013) and in the teleost trigeminal ganglion (Kerem et al., 2005). (For a helpful pictorial overview of the segregation of opV/profundal, maxillary and mandibular trigeminal neurons in various vertebrate groups, see Fig. 2 in Kerem et al., 2005.) In the mouse, the spatial segregation of maxillary and mandibular neurons is established before axon outgrowth, i.e., before any contact with peripheral targets (Erzurumlu and Jhaveri, 1992; Scott and Atkinson, 1999; Hodge et al., 2007). Indeed, expression of the transcription factor *Hmx1* is restricted to neurons in the mandibular (caudal-most) portion of the mouse trigeminal ganglion as early as E9.5 (Hodge et al., 2007) (also see Erzurumlu et al., 2010). Moreover, the existing fate-map data cannot rule out the possibility that maxillary and mandibular trigeminal neuron precursors are spatially segregated within the mmV

placode in gnathostomes. The recent detailed fate-map of *Xenopus* neurogenic placodes did not track labeled cells through to ganglion stages (Pieper et al., 2011). In chick, where ganglion stages were examined, the quail-chick grafting approach was probably insufficiently fine-grained (D'Amico-Martel and Noden, 1983), while after focal DiI labeling it would have been very difficult to distinguish maxillary versus mandibular neurons on transverse sections without any markers, even had the possibility of spatial segregation been considered when these experiments were performed (Xu et al., 2008). Whether the gnathostome mmV placode is in fact bipartite, with segregated precursors for maxillary and mandibular trigeminal neurons, remains an intriguing possibility for future research. Unfortunately, no cross-species molecular markers for mmV placode cells have as yet been identified, in contrast to opV placode cells, which express Pax3 in all gnathostomes (Stark et al., 1997; Baker et al., 1999; Schlosser and Ahrens, 2004; O'Neill et al., 2007; Modrell et al., 2011). As shown here by immunostaining with a pan-Pax3/7 antibody (Davis et al., 2005), lamprey opV neurons also express the single lamprey *Pax3/7* gene (McCauley and Bronner-Fraser, 2002; Osório et al., 2005; Kusakabe et al., 2011), confirming Pax3 as a pan-vertebrate marker for opV placode-derived neurons.

### **Lamprey cranial sensory ganglia contain neural crest-derived cells**

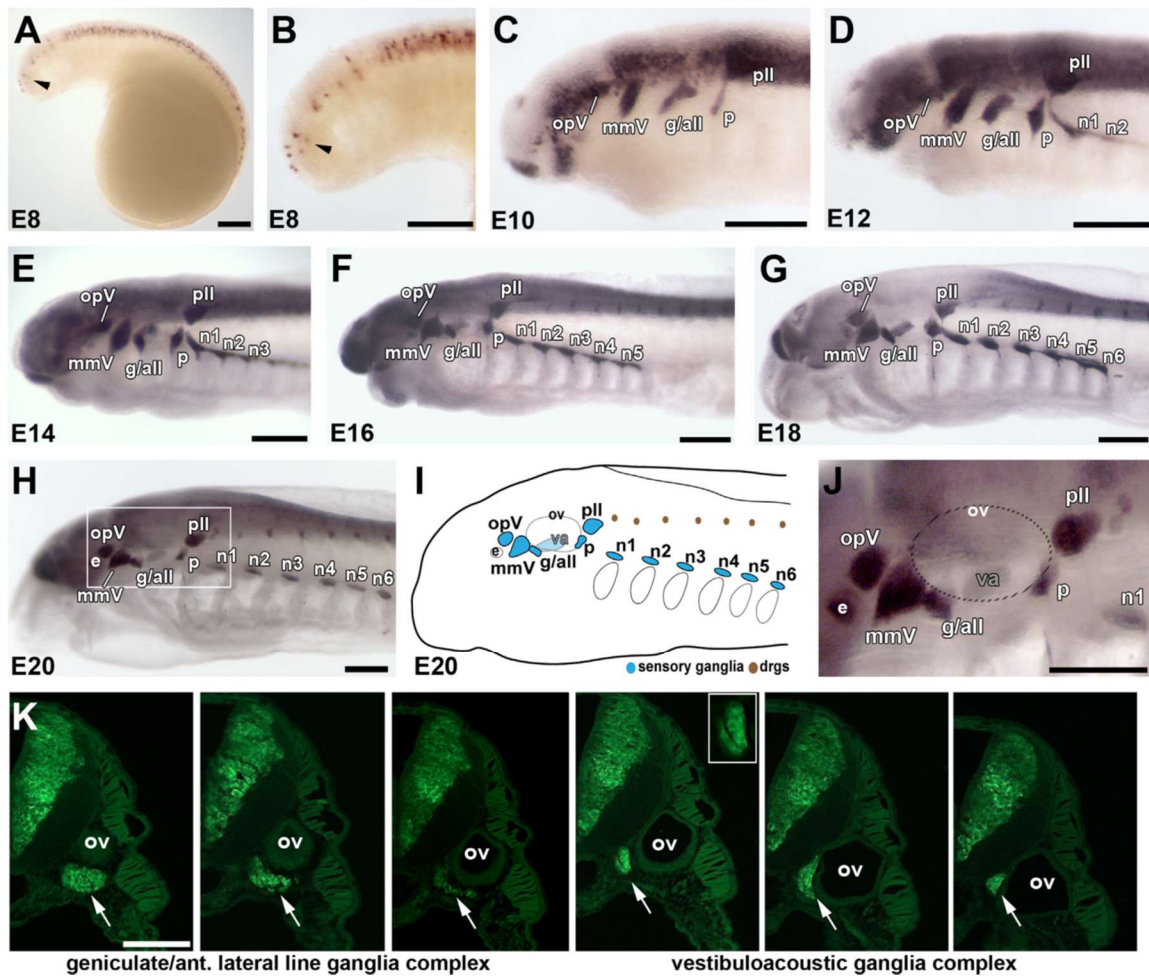
A previous fate-mapping study in *P. marinus* in which premigratory neural crest cells were DiI-labeled at approximately E6 demonstrated a neural crest contribution to the branchial arches but not to the cranial sensory ganglia, unless ectoderm was also labeled (McCauley and Bronner-Fraser, 2003). Furthermore, the expression of a lamprey homolog of *Sox10*, which in gnathostomes is expressed in migrating neural crest cells and maintained



in the peripheral glial lineage (see Britsch et al., 2001), was excluded from developing cranial ganglia (McCauley and Bronner-Fraser, 2003). The authors suggested that the neural crest contribution to the cranial sensory ganglia may have arisen within the gnathostome lineage. This is surprising because in gnathostomes, cranial neural crest cells not only give rise to somatosensory neurons in the trigeminal ganglia and proximal (“root”) ganglia of other cranial nerves, but also to the satellite glia of all cranial sensory ganglia (e.g. Yntema, 1943; Yntema, 1944; Hamburger, 1961; Narayanan and Narayanan, 1980; Ayer-Le Lièvre and Le Douarin, 1982; D'Amico-Martel and Noden, 1983; Kious et al., 2002; Harlow et al., 2011; Quina et al., 2012). A neural crest contribution to lamprey cranial sensory ganglia is supported by their reduction in lamprey embryos in which the function of various neural crest specifier genes (e.g. from the *Msx*, *Zic*, *Id* and *FoxD3* gene families) was knocked down using anti-sense morpholinos (Sauka-Spengler et al., 2007). Here, we labeled cranial neural crest cell precursors at E5-6 (up to 24 hours earlier than McCauley and Bronner-Fraser, 2003) and observed DiI-positive cells migrating away from the injection site to contribute to several cranial sensory ganglia, including the mmV and nodose ganglia, as well as scattered cells along their nerve fibers (presumptive Schwann cells; Nakao and Ishizawa, 1987). We suggest that differences in the time of injection may explain the lack of DiI-labeled neural crest cells in the cranial sensory ganglia in previous fate-mapping experiments (McCauley and Bronner-Fraser, 2003). Overall, we conclude that the mechanisms underlying the development of neurogenic placodes and cranial sensory ganglia in the lamprey are likely to be highly conserved with gnathostomes.

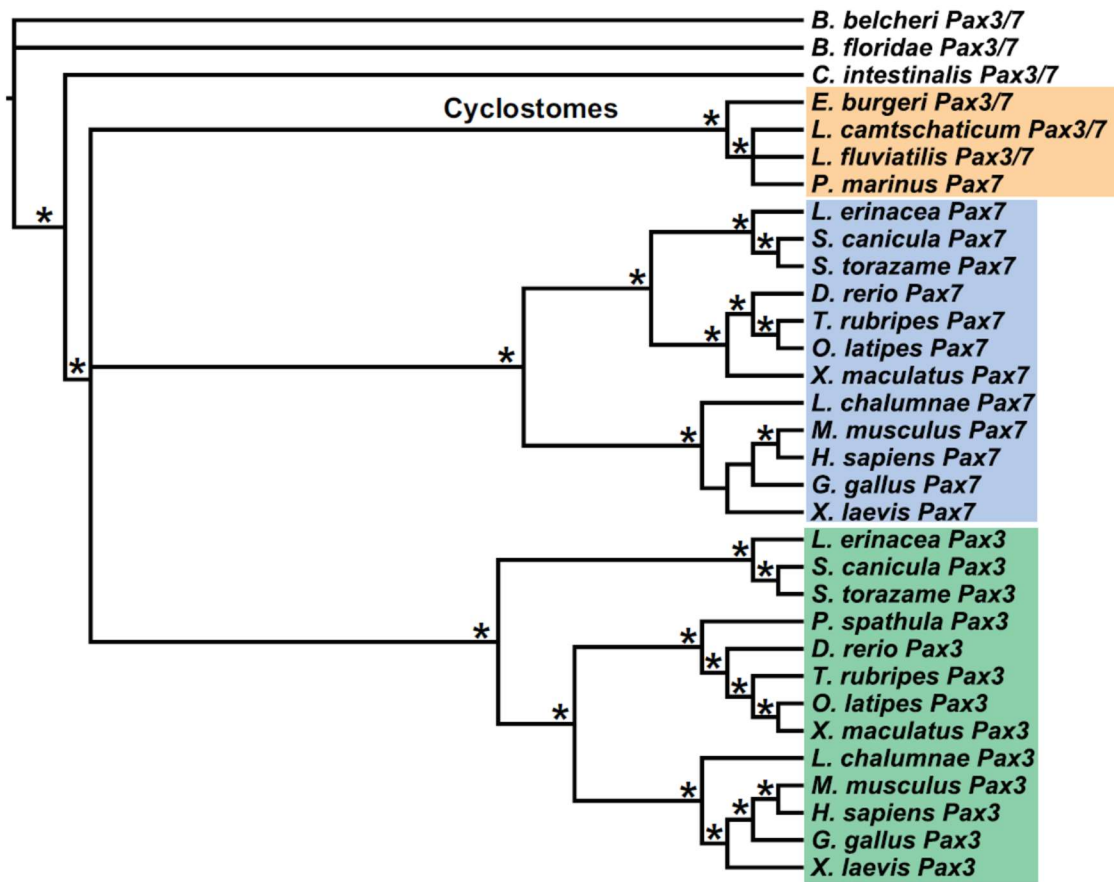
**Acknowledgments**

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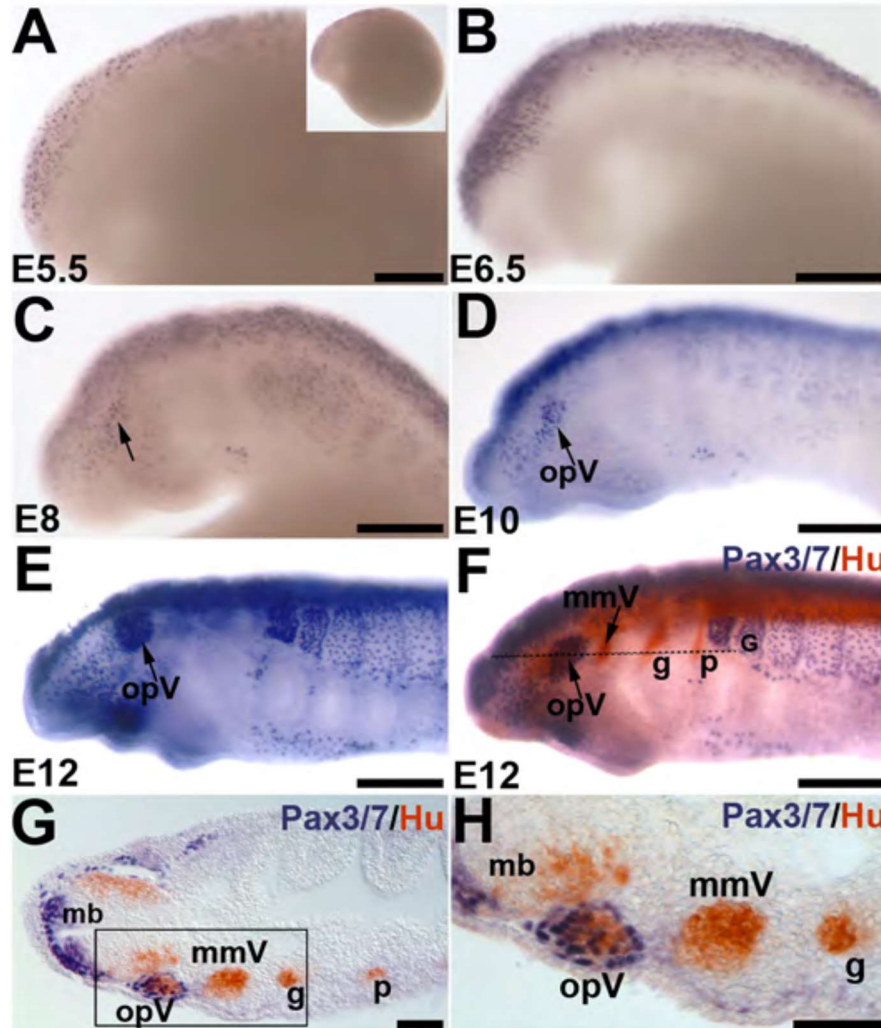


**Fig. 1. Spatiotemporal development of lamprey cranial sensory ganglia.** Anterior is to the left for all whole-mount images. (A,B) Low-power (A) and higher-power view (B) of an embryo at embryonic day 8 (E8) immunostained for the pan-neuronal Elav-family members HuC/D (Hu). HuC/D expression is strong in neurons within the neural tube, with fainter expression in neurons lateral to the rostral neural tube (arrowhead). (C) By E10, discrete lateral patches of HuC/D expression reveal the primordia of all cranial sensory ganglia except the nodose: the ophthalmic trigeminal ganglion (opV), the maxillomandibular trigeminal ganglion (mmV), the geniculate/ant. lateral line ganglionic complex (g/all), the petrosal ganglion (p) and the posterior lateral line ganglion (pll). (D-G) HuC/D immunostaining of embryos at E12 (D), E14 (E), E16 (F) and E18 (G) shows the development of the six nodose ganglia in a rostrocaudal sequence dorsal to the branchial arches and the progressive condensation of all the cranial ganglia. Dorsal root ganglia are also visible from E16, adjacent to the dorsal neural tube. (H-J) By E20, all the cranial sensory ganglia have formed. (H) Low-power and (I) schematic view of an E20 embryo, showing the location of cranial sensory ganglia (blue in I) and dorsal root ganglia (brown in I). (J) A higher-power view of the boxed area in H, showing distinct opV and mmV ganglia, the geniculate/ant. lateral line ganglionic complex, the vestibuloacoustic ganglion (medial to the otic vesicle, hence hardly stained in whole-mount), the petrosal ganglion, the posterior lateral line ganglion and the most rostral nodose ganglion (n1). (K) Transverse serial

sections immunostained for HuC/D (green), starting near the rostral edge of the otic vesicle (see panel J for orientation of otic vesicle, which is indicated by a dotted oval) and progressing caudally through the geniculate/aLL ganglionic complex ventral to the otic vesicle (arrow, left-hand three images) and then the vestibuloacoustic ganglion medial to the otic vesicle (arrow, right-hand three images). In the fourth and fifth images, the developing intracapsular ganglion (second ganglion of the anterior lateral line nerve) may also be visible, medial to the vestibuloacoustic ganglion and slightly separated from it by a thin HuC/D-negative space (inset). Abbreviations: all, anterior lateral line ganglion; drgs, dorsal root ganglia; e, eye; g, geniculate ganglion; mmV, maxillomandibular trigeminal ganglion; n, nodose ganglion; opV, ophthalmic trigeminal (profundal) ganglion; ov, otic vesicle; p, petrosal ganglion; pll, posterior lateral line ganglion; va, vestibuloacoustic ganglion. Scale bars: A-J 0.2 mm; K 50  $\mu$ m.

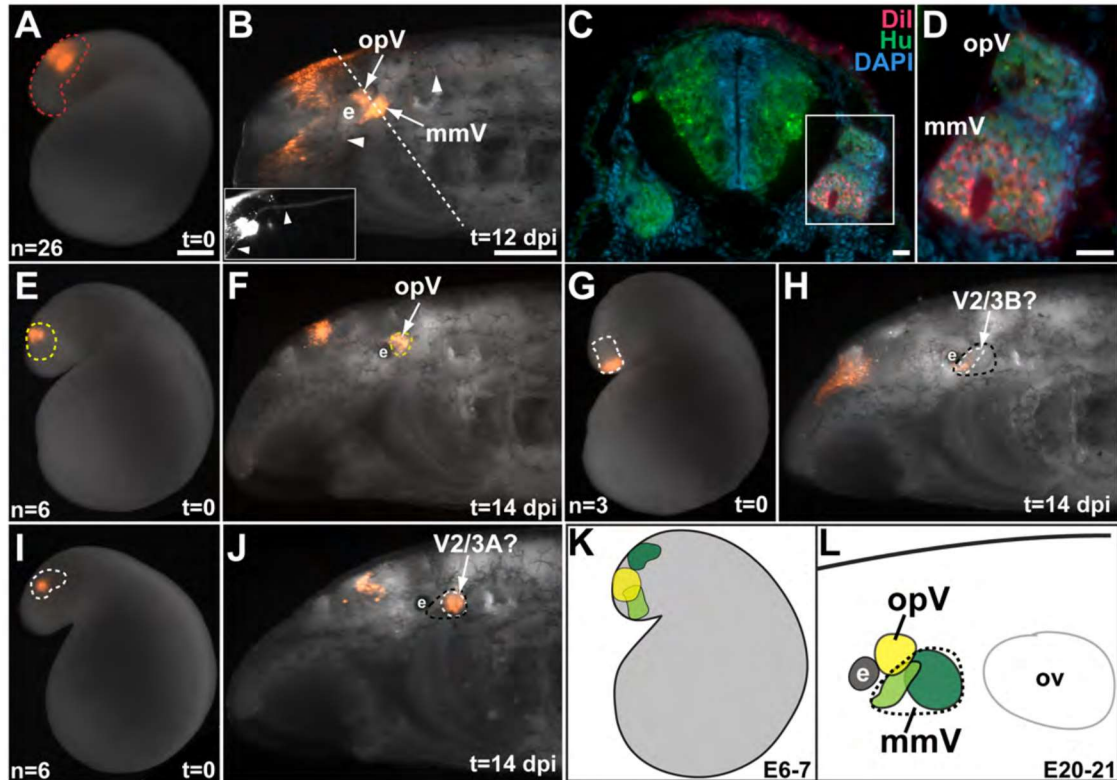


**Fig. 2. Cyclostome Pax3/7 subfamily proteins form a well-supported clade, separate from gnathostome Pax3 and Pax7 clades.** Maximum *a posteriori* (MAP) topology, obtained with Bali-Phy software, summarizing all of the phylogenetic analyses performed. Nodes consistently recovered with high support in all Bayesian and coalescence-based analyses are indicated by an asterisk (where the posterior probability is > 90). These analyses consistently recovered three main clades of orthologous sequences: (i) a cyclostome clade containing all published hagfish and lamprey Pax3/7 proteins (orange box), (ii) a gnathostome Pax7 clade (blue box) and (iii) a gnathostome Pax3 clade (green box). Relationships between these three clades, however, remain unresolved: the gnathostome Pax3 and Pax7 clades formed a sister group in most analyses but with low support. Within the gnathostome clades, relationships are well resolved for the Pax3 but not the Pax7 subfamily: in some cases, short sequence lengths for Pax7 likely account for a topology that is incongruent with the currently proposed relationships among vertebrates. Generally, however, phylogenetic relationships recovered here are consistent with current understanding of the chordate phylogeny.



**Fig. 3. Pax3/7 is a specific marker for opV placode-derived neurons.** Whole-mount immunostaining with the pan-Pax3/7 antibody DP312 (Davis et al., 2005) shows (A) Pax3/7 expression at E5.5 in the anterior-most dorsal neural tube; inset shows entire embryo. (B) By E6.5, more Pax3/7-positive cells appear along the length of the neural tube and in presumptive migrating neural crest cells adjacent to it. (C) At E8, Pax3/7 expression is seen in the neural tube, somites and scattered cells lateral to the neural tube (arrow). (D) By E10, a patch of Pax3/7-positive cells (arrow) is seen lateral to the neural tube in a similar position to the developing opV ganglion (compare with Fig. 1C). (E) By E12, Pax3/7 is strongly expressed in the presumptive opV placode/ganglion (opV, arrow); (F) this is confirmed by double immunostaining for Pax3/7 (blue) and the pan-neuronal marker HuC/D (red). Dotted line shows plane of section in G,H. (G) Coronal section shows Pax3/7 expression in the dorsal neural tube and opV ganglion, but not in the mmV, geniculate or petrosal ganglia. (H) A higher-power view of the boxed region in E confirms that Pax3/7 expression is restricted to the developing opV ganglion (and dorsal neural tube cells). Abbreviations: g, geniculate ganglion; mb, midbrain; mmV, maxillomandibular trigeminal ganglion; opV, ophthalmic trigeminal placode/ganglion; p, petrosal ganglion. Scale bars: A-F 0.2 mm; G, H 50  $\mu$ m.



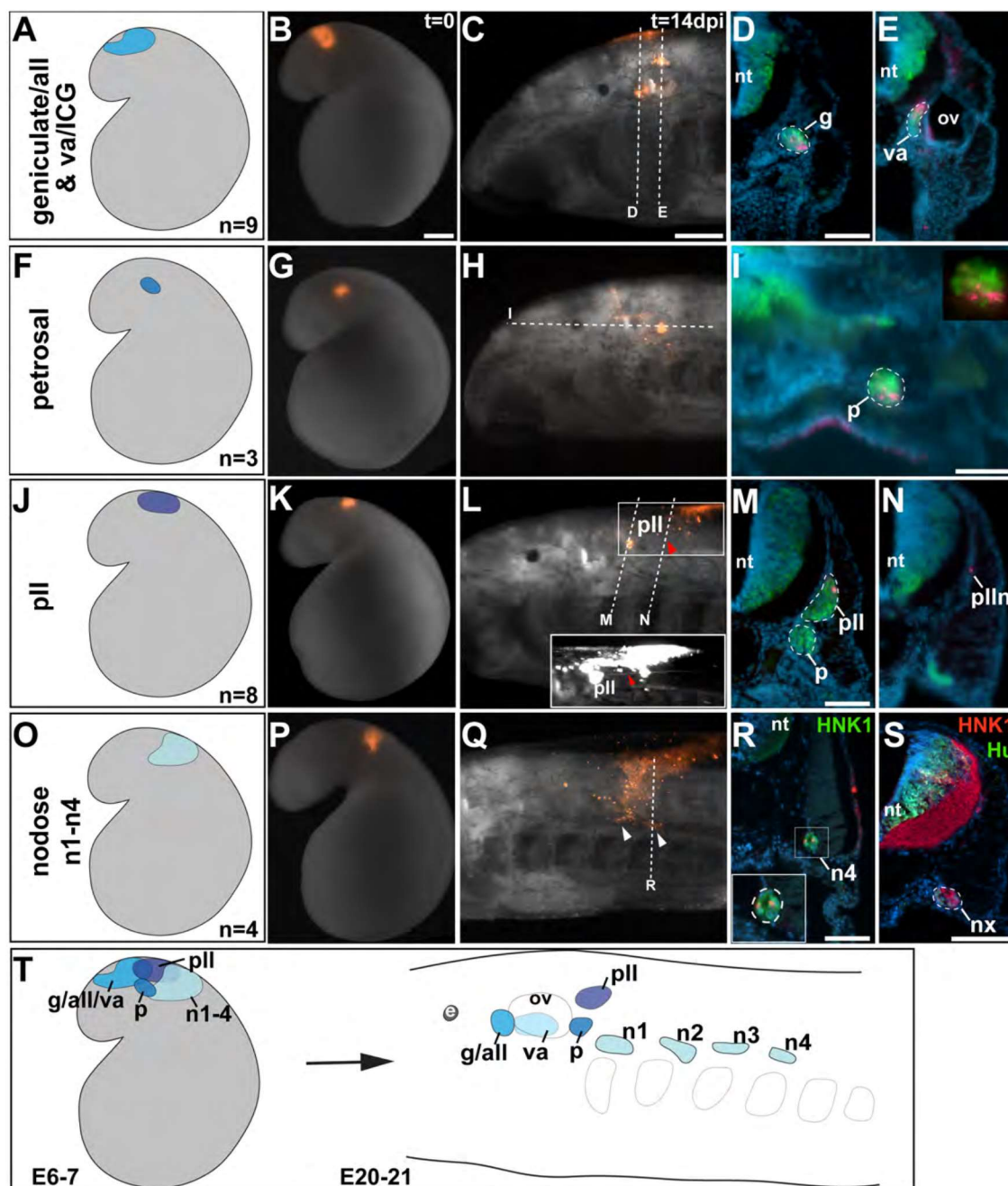


**Fig. 4. Fate-mapping of opV and mmV placode-derived neurons reveals two separate domains of mmV neuron precursors at late neurula stages.** Anterior is to the left for all whole-mount images. (A) An E6.5 embryo immediately after DiI injection into a broad patch of anterodorsal head ectoderm within the region outlined in red, which contains both opV and mmV neuron precursors. (B) The same embryo as in A at E18 (t=12 dpi), showing DiI labeling in the opV and mmV ganglia. DiI is also visible in the upper lip-innervating mmV nerve (V2/3A, inset, ventral arrowhead) and central projections from the mmV ganglion (inset, dorsal arrowhead). Dotted line shows plane of section in C, D. (C) Merged images of low-power and (D) higher-power views of an oblique section through both the opV and mmV ganglia, immunostained for the neuronal marker HuC/D (green) and counterstained for the nuclear marker DAPI (blue), showing DiI (red) in surface ectoderm and also co-localized with neurons (HuC/D, green) in both the opV and mmV ganglia. (E) An E6.5 embryo immediately after focal DiI injection into the region of head ectoderm outlined in yellow, which contains opV neuron precursors. (F) The same embryo as in E at E20 (t=14 dpi), showing restriction of DiI to the opV ganglion. (G) An E6.5 embryo immediately after a focal DiI injection into the region of head ectoderm outlined in white (shaded light green in schematic). (H) The same embryo as in G at E20 (t=14 dpi), showing DiI localization to a relatively small, rostral domain (outlined in white) of the mmV ganglion (outlined in black), which may correspond to lower lip/velum-innervating V2/3B neurons (see text). (I) An E6.5 embryo immediately after a focal DiI injection into the region of head ectoderm outlined in white (shaded dark green in schematic). (J) The same embryo as in I at E20 (t=14 dpi), showing DiI localization to a larger, caudal domain (outlined in white) of the mmV ganglion (outlined in black), which may correspond to upper lip-innervating V2/3A neurons (see text). (K) Schematic fate-map at E6-7 summarizing the location of opV neuron precursors (yellow) between two separate patches of mmV neuron precursors (light and dark green). (L) Schematic summarizing the fate at E20-21 of DiI-injected cells within the locations shown in panel K. The

opV ganglion (yellow) lies dorsal to the mmV ganglion (dotted black outline). Rostral (light green) and caudal (dark green) subregions of the mmV ganglion are distinguishable in the E6-7 fate-map, which may correspond to V2/3B and V2/3A neurons, respectively (see text).

Abbreviations: dpi, days post-injection; e, eye; mmV, maxillomandibular trigeminal ganglion; opV, ophthalmic trigeminal ganglion; ov, otic vesicle; t, time, V2/3A, upper lip-innervating trigeminal neurons; V2/3B, lower lip/velum-innervating trigeminal neurons. Scale bars: A, E, G, I 0.2 mm; B, F, H, J 0.2 mm; C, D 10  $\mu$ m.

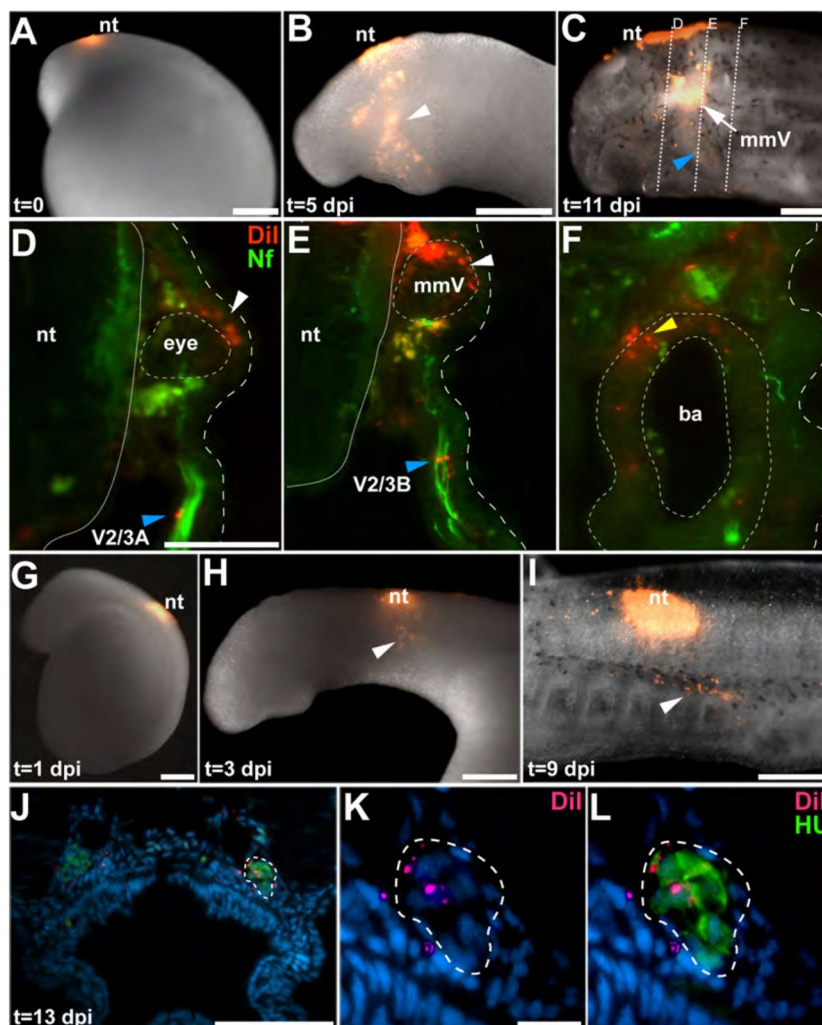




**Fig. 5. Fate-maps at E6-7 for lamprey epibranchial and lateral line placode-derived ganglia.**

(A-E) Ectoderm in the coloured region in (A) was fated to contribute to neurons in the geniculate/anterior lateral line (aLL) ganglionic complex and, in 7/9 cases, also to the vestibuloacoustic/intracapsular ganglionic complex. (B) An embryo shortly after DiI injection at E6.5 (t=0) in the region shown in A. (C) The same embryo as in B, at E20 (t=14 dpi), with DiI visible in the geniculate/aLL complex. Dotted lines indicate planes of section in D, E. (D, E) Transverse sections through (D) the geniculate/aLL ganglionic complex and (E) the vestibuloacoustic ganglion, immunostained for the neuronal marker HuC/D (green) and counterstained with DAPI (blue), showing co-localization of DiI with HuC/D. (F-I) Ectoderm in

the coloured region in (F) was fated to contribute to neurons in the petrosal ganglion. (G) An embryo shortly after DiI injection at E6.5 ( $t=0$ ) in the region shown in F. (H) The same embryo as in G, at E20 ( $t=14$  dpi), showing DiI in the petrosal ganglion. Dotted line indicates plane of section in I. (I) Coronal section through the petrosal ganglion, showing co-localization of DiI and HuC/D. (J-N) Ectoderm in the coloured region in (J) was fated to contribute to neurons in the posterior lateral line (pLL) ganglion. (K) An embryo shortly after DiI injection at E6.5 ( $t=0$ ) in the region shown in J. (L) The same embryo as in K, at E20 ( $t=14$  dpi), showing DiI in the pLL ganglion and the pLL nerve (inset; red arrowheads). (M) Transverse section through the petrosal and pLL ganglia showing DiI specifically in the pLL ganglion. (N) Transverse section further caudally showing DiI in the pLL nerve. (O-R) Ectoderm in the coloured region in (O) was fated to contribute to neurons in the nodose ganglia. (P) An embryo shortly after DiI injection at E7 ( $t=0$ ) in the region shown in O. (Q) The same embryo as in P, at E21 ( $t=14$  dpi), showing DiI in the third and fourth nodose ganglia (arrowheads). Dotted line indicates plane of section in R. (R) Transverse section through the fourth nodose ganglion (n4), in this case immunostained for HNK1 (green). Inset shows higher-power view of the ganglion and co-localization of DiI (red) with HNK1 immunoreactivity (green). (S) Transverse section through one of the nodose ganglia in a control embryo immunostained for HNK1 (red) and HuC/D (green), confirming expression of HNK1 in lamprey sensory ganglia. (T) Schematic summary of the fate-map for epibranchial and lateral line placode-derived ganglia at E6-7: the different regions that gave rise to neurons in the corresponding ganglia at E20-21 are indicated in varying shades of blue. Abbreviations: all, anterior lateral line; dpi, days post-injection; e, eye; g, geniculate; ICG, intracapsular ganglion; n, nodose; nt, neural tube; ov, otic vesicle; p, petrosal; pll, posterior lateral line; plln, posterior lateral line nerve; t, time; va, vestibuloacoustic. Scale bars: B, G, K, P 0.2 mm; C, H, L, Q 0.2 mm; D, E, I, M, N, R, S 50 $\mu$ m.



**Fig. 6. Neural crest-derived cells are found in cranial sensory ganglia and along cranial nerves (presumptive Schwann cells).** (A) An E5 embryo immediately after DiI injection (t=0 dpi) into the presumptive rostral hindbrain. (B) At E10 (t=5 dpi), labeled neural crest cells are observed in optic, trigeminal and mandibular arch regions (arrowhead). (C) At E16 (t=11 dpi), DiI labeling is seen in the mmV ganglion (arrow) and on the lower lip/velum-innervating mmV nerve branch (V2/3B, arrowhead). Dotted lines indicate planes of section in D-F. (D-F) In transverse sections immunostained for neurofilament (green), DiI (red) is observed in neural crest-derived cells (D) around the eye (white arrowhead) and on the upper lip-innervating mmV nerve branch (V2/3A, blue arrowhead); (E) in the mmV ganglion (white arrowhead) and on the lower lip/velum-innervating mmV nerve branch (V2/3B, blue arrowhead). (F) As expected, DiI labeling is also observed within the neural crest-derived branchial arch basket (yellow arrowhead). (G) An E6.5 embryo one day after DiI injection (t=1 dpi) at late E5 into the dorsal neural tube in the vagal region. (H) The same embryo as in G at E9 (t=3 dpi). DiI-labeled neural crest cells are observed migrating ventrally (arrowhead). (I) The same embryo at E15 (t=9 dpi), showing DiI-labeled neural crest cells (arrowhead) dorsal to the branchial arches. (J-L) At E19 (t=13 dpi), immunostaining on transverse sections through the nodose ganglia for the neuronal marker HuC/D (green), counterstained with DAPI (blue), revealed DiI-positive cells (red) in the nodose ganglia (J, lower-power view; K,L, higher-power views). Abbreviations: ba, branchial arch basket; dpi, days post-injection; mmV, maxillomandibular trigeminal ganglion; nt, neural tube; t, time; V2/3A, upper lip-innervating mmV nerve branch; V2/3B, lower lip/velum-innervating mmV nerve branch. Scale bars: A-C, G-I 0.2mm; D-F, J 50  $\mu$ m; K, L 10  $\mu$ m.

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